Chapter 19

Distribution, Mechanisms, Impact and Management of Insecticide Resistance in Malaria Vectors: A Pragmatic Review

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1. Introduction

Malaria is still a major burden causing the death of nearly 655,000 people each year, mostly in children under the age of five, and affecting those living in the poorest countries [1]. Currently, the major obstacles to malaria control and elimination are the absence of a protective vaccine, the spread of parasite resistance to anti-malarial drugs and the mosquito resistance to insecticides [2]. Controlling mosquito vectors is fundamental to reduce mosquito-borne diseases by targeting vectorial capacity and hence the transmission. Vector control through the use of chemicals for mosquito bed nets and indoor residual spraying is still the cornerstone of malaria prevention [1]. Unfortunately, the extensive use of insecticides since the 1950s has led to the development of strong resistance worldwide hence representing a major public health problem where insecticidal vector control is implemented. Here, we propose to review the current level, distribution and mechanisms of insecticide résistance in malaria vectors and address their impact on the efficacy of vector control interventions. Strategies to prevent and/or delay the spread of insecticide resistance in natural mosquito populations are also discussed.

2. Definition of resistance

According to the *World Health Organization* (WHO), resistance is defined as the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations [3]. This definition differs from that provided by the *Insecticide Resistance Action committee* (IRAC) (www.irac-online.org) that gathers independent scientists and experts belonging to Agrochemical Companies who define operational (field) resistance as a heritable change in the sensitivity of a pest population that is reflected in the

repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species. The IRAC definition, although pragmatic, is less "sensitive" with the scope to implement early *Insecticide Resistance Management* (IRM) strategies in the field. In both cases however, appropriate tools (biological, biochemical and/or molecular) are needed to identify the mechanisms involved and to conduct surveillance at individual and/or population levels [4].

Resistance has been observed in more than 500 insect species worldwide among which more than 50 *Anopheles* species (Diptera: Culicidae) are responsible for the transmission of malaria parasites to humans [5]. Resistance is a heritable character that relies on a genetic basis. Resistance results from the selection of a genetic modification in one or several genes occurring by migration and/or mutation. For example, when a mosquito population is exposed to an insecticide A, the individuals having resistant genes to this insecticide A survive and reproduce until the resistant allele becomes almost fixed. The use of insecticides for agricultural purposes and more recently for public health has played pivotal step in the selection of resistance in malaria vectors [6]. Resistance can involve several physiological and/or behavioural changes. Changes in the insecticide target site that reduce its binding to insecticides (known as target-site resistance) is the best understood type of resistance mechanism and molecular diagnostics to detect this resistance mechanism are now integrated into insecticide resistance monitoring strategies in malaria control programmes [7, 8]. Enhanced insecticide metabolism that lowers the amount of insecticide reaching the target site (known as metabolic resistance) is more complex but recent advances have identified key enzymes responsible for insecticide detoxification, paving the way for the development of molecular markers for this type of resistance mechanism [9, 10]. Other physiological changes (e.g. reduce penetration through cuticular resistance) and/or behavioural changes in the mosquito population were identified but their impact on the efficacy of insecticides is still poorly understood.

lt is commonly accepted that the enhanced metabolism and target site modifications are responsible for high level of insecticide resistance in malaria vectors. To date, malaria vectors have developed resistance to the main chemical classes used in public health (i.e. pyrethroids, DDT, carbamates and organophosphates) (table 1) and the occurrence of cross-resistance[1] and multiple resistance[2] represent a serious threat to achieving the Millennium Development Goals for malaria control (i.e 75% reduction of global malaria cases by 2015). Surveillance and routine monitoring campaigns to assess the level and type of resistance are essential to help Malaria Control Programme (MCPs) to design more effective and sustainable malaria vector control strategies at an operational scale [4].

3. History of resistance to public health insecticides

Since the humans used chemicals for crop protection and/or the prevention of vector borne diseases, cases of resistances have been reported [11, 12]. Insecticides used for malaria control

1**Cross resistance:** occurs when a resistance mechanism, which allows insects to resist one insecticide, also confers resistance to another insecticide. Cross resistance can occur between insecticides from different chemical classes.

2**Multiple resistance:** occurs when insects develop resistance to several compounds by expressing multiple resistance mechanisms. The different resistance mechanisms can combine to provide resistance to multiple classes of products.

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have included organochlorine, organophosphorus, carbamate, and pyrethroid insecticides, with the latter now taking increasing market share for both indoor residual spraying and Long Lasting Insecticidal mosquito Nets (LLINs) programmes [13]. Resistance has naturally tended to follow the use and switches of these insecticides [5].

Insecticides	Molecular target	Resistance mechanisms		References
		target site	enzymatic mechanisms	
Pyrethroids, type I	sodium channel	Kdr and super Kdr mutations	monooxygenases + esterases	[10,61,62]
Pyrethroids, Type II	sodium channel	Kdr mutations	monooxygenases + esterases	[10,61,62]
Organochlorates	sodium channel	Kdr mutations	GS-tranferases + monooxygenases	[10,61,62]
N-alkyl-amides	sodium channel	no resistance reported against insect of public health importance		
Organophosphates	acetylcholinesterase	$Ace1^R$ mutation	esterases + GS-tranferases + monooxygenases	[10,59,63-67]
Carbamates	acetylcholinesterase	$Ace1^R$ mutation		[10,59,63-67]
Neonicotinoids	nicotinic acetycholine receptor	not reported	monooxygenases	[68,69]
Spinosad	nicotinic acetycholine receptor	not reported		
Cyclodienes, Lindane, Bicyclic phosphates	GABA receptor	Rdl mutation	GS-transferases	[62,70]
Phenylpyrazoles	GABA receptor	Rdl mutation	GS-transferases	[62,70]
Avermectines	GABA receptor	undiscribed	monooxygenases + esterases	[62,70]
Insect Growth Regulators	Ecdysone agonist/disruptor or inhibitor of ATP synthase, chitin biosynthesis or lipid synthesis	no resistance reported against insect of public health importance		
Bacillus thuringiensis var. israelensis	microbial disruptors of insect midgut membranes	reported against Culex pipiens s.l. but non discribed		[71]

Table 1. Mechanisms of insect resistance to the main insecticide families of public health interest

Historically, DDT was first introduced for mosquito control and malaria eradication programme in 1946. The first case of DDT resistance was reported in *An. sacharovi* in Greece in 1953 and was followed by dieldrin resistance in 1954 [15]. Onset of resistance was marked by deterioration in malaria control that has continued for more than 30 years with sporadic epidemics of disease [16]. Resistance in *An. sacharovi* has been later reported in Bulgaria, Lebanon, Iran, Iraq, and Syria [12]. Pronounced DDT resistance appeared in *An. stephensi* in Iran and Iraq when full scale house spraying operations began in 1957 and dieldrin resistance appeared three years later. In India, house-spraying of DDT and Lindane (HCH) under the public health programme was introduced in the 1950s. Resistance of the main malaria vector *An. culicifacies* to dieldrin developed in 1958 [17] and resistance to DDT in 1959 [18], but the malaria control programme continued until 1965-1966 when both DDT and HCH failed to control outbreaks of malaria [19]. As a result, malathion was introduced in some areas in 1969 with some success but *An. culicifacies* rapidly developed resistance by 1973 [20]. Malathion resistance resulted in colossal epidemics of malaria in 1975 with 4 million cases reported as compared with 125,000 in 1965. The experience in Pakistan was similar with DDT resistance appearing in 1963. The importance of the resistance was not recognized until outbreaks of malaria began in 1969 and neither DDT nor HCH was effective. By 1975, malaria cases were reported in Pakistan to number 100 million as compared to 9,500 in 1961 [12]. DDT resistance in *An. culicifacies* was reported in Sri Lanka in 1968 resulting in a severe epidemic of malaria

[21]. This vector is now resistant to DDT, dieldrin, organophosphates, carbamates and pyrethroids [11].

Similar trend was noted in Central America and the Caribbean. Dieldrin spraying against *An. albimanus* begun in 1956 and widespread resistance appeared in 1958 [12]. A return to DDT spraying produced generalized resistance by 1960 [18]. The carbamate propoxur was employed in El Salvador, Guatemala, Honduras and Nicaragua in 1970 and resistance developed by 1974. *An albimanus* now exhibits multiple resistances to DDT, dieldrin, lindane and other chemical recently used in public health [22].

Much less information is however available for South East Asian malaria vectors, most probably because resistance monitoring was not carried out in routine before the 80s. In Vietnam, DDT resistance was found in 1989 in *An. epiroticus* of the Sundaicus Complex and is still occurring [23]. From 1990 till 2000, pyrethroid resistance was almost absent in all tested species except in some populations of *An. vagus* and *An. minimus s.l.* [24]. In Thailand, no evidence of insecticide resistance in malaria vectors was present before 1985 [25]. In 1986, development of physiological resistance to DDT was detected in *An. aconitus* from the north where DDT was commonly used for malaria control. One year later, DDT resistance was found in field collected mosquitoes of *An. philippinenis*, *An. nivipes* and *An. aconitus* from the same northern region. Between 1990 and 1997, DDT resistance has been detected in the three primary malaria vectors *An. dirus s.l.*, *An. minimus s.l.* and *An. maculatus s.l.* and permethrin resistance was suggested in a population of *An. minimus s.l.* from northern Thailand, based however on a lower discriminative dosage (0.25%) of permethrin than that used today [25].

In Africa, resistance was initially found in *An. gambiae* in Bobo Dioulasso by 1967 (Burkina Faso), hence less than 7 years after the end of DDT use for malaria control [12]. DDT resistance was found in neighbouring countries including Cote d'Ivoire, Nigeria and Mali [26] and was then reported in most of Central and East African countries [27]. Strong association was observed between the level of DDT resistance in malaria vectors and the amount of DDT use for cotton protection [28]. Regarding BHC/dieldrin, the first cases of resistance were reported in Nigeria in 1954 hence only few months after the introduction of this molecule for malaria control. Initially found in very limited geographical areas, dieldrin resistance has spread in areas free of any insecticide treatments [29]. Few years later, resistance was reported in Bobo-Dioulasso and Cote d'Ivoire [30]. Today, resistance to BHC/dieldrin is still widespread in wild field anopheline populations despite its abandon in public health for many decades [31]. As for DDT, dieldrin resistance in malaria vectors arose and persisted from intensive use of pesticide for agricultural practices and in some specific settings due to public health programmes [32, 33].

After the 80s, DDT has been more or less abandoned worldwide and replaced by organophosphate (OP), pyrethroids and, to lesser extent, carbamates. However, insecticide resistance continued to be a problem, and vector control operations were affected, particularly in India, Africa and Latin America, by extensive use of agricultural pesticides. OP resistance, either in the form of broad-spectrum OP resistance or malathion-specific resistance was found in the major malaria vector species worldwide [12]. Pyrethroids were introduced in late 70s in public health and increasingly used in the 90s; however, cases of resistance were rapidly reported in

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the main malaria vectors worldwide including *An. albimanus* [34], *An. darlingi* [35], *An. culicifacies* [36], *An. stephensi* [37], *An. gambiae* [38], *An. funestus* [39] and *An minimus* [40]. Despite a sporadic use (compared to DDT and pyrethroids), resistance to carbamates was earlier reported in several mosquito species including *An. albimanus* [41], *An. atroparvus* [42], *An. sacharovi* [5] and *An. gambiae* [43]. Carbamate resistance is now spreading in malaria vectors especially in West Africa where it has been reported in Cote d'Ivoire [44], Burkina Faso [45, 46], Benin [47] and Nigeria [48]. Increased level of carbamate resistance in African mosquito populations is worrying for malaria control because these chemicals are increasingly used in replacement to pyrethroids for Indoor Residual Spraying (IRS) [49].

It is obvious that insecticide resistance in malaria vectors is increasing worldwide due to the increasing selection pressure on mosquito populations caused by the presence of urban, domestic and/or agricultural pollutants in the environment [50]. Transversal and longitudinal monitoring surveys are essential to address the spatio-temporal changes in resistance (dynamic) and to design appropriate strategies for a better control of resistant malaria vector populations worldwide.

4. Resistance mechanisms

The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories including metabolic resistance, target-site resistance, reduce penetration and behavioral avoidance. These mechanisms that are shown in the figure 1 are briefly described in the following sections.

4.1. Metabolic resistance

Metabolic resistance is the most common resistance mechanism that occurs in insects. This mechanism is based on the enzyme systems which all insects possess to help them to detoxify naturally occurring xenobiotics/insecticides. It is commonly accepted that insect detoxification systems derived from the plant-insect evolutionary arm race and several insect detoxification enzymes have been associated to the detoxification of plant toxins and all types of chemicals, including insecticides [51]. Over-expression of enzymes capable of detoxifying insecticides or amino acid substitutions within these enzymes, which alter the affinity of the enzyme for the insecticide, can result in high levels of insecticide resistance (see [52] for review). Increased expression of the genes encoding the major xenobiotic metabolizing enzymes is the most common cause of insecticide resistance in mosquitoes. Over expression of detoxyfing enzymes can occur as the result of gene amplification (e.g. duplication) or due to changes in either trans-acting regulator elements or in the promoter region of the gene [5, 53, 54]. The consequence is a significant increase of enzyme production in resistant insects that enables them to metabolize or degrade insecticides before they are able to exert a toxic effect. Three categories of enzymes, namely esterases, P450s and glutathione-S-transferases are known to confer resistance to insecticides in insect pest such as malaria vectors. These large enzyme families contain multiple enzymes with broad overlapping substrate specificities, and one member of the family might

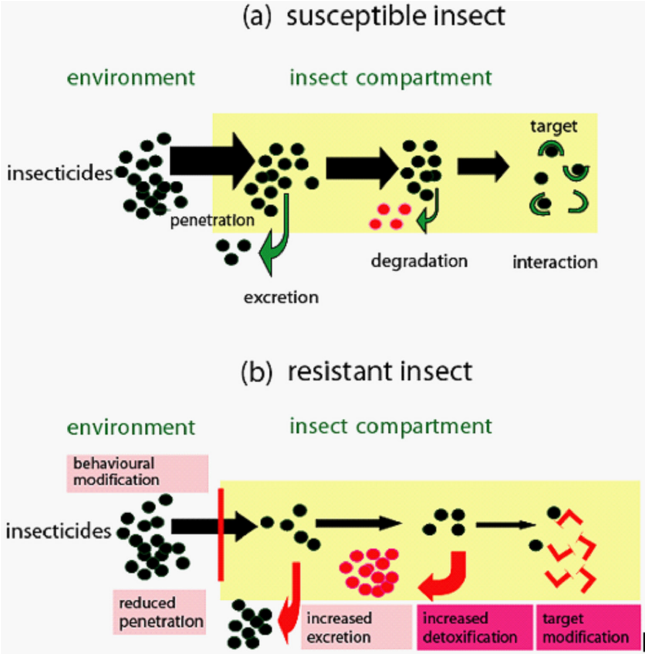

Figure 1. Scheme of potential behavioral and physiological changes associated with insecticide resistance in malaria vectors; (a) susceptible insect; (b) resistant insect *(source ; see [14])*

be capable of metabolizing limited number of insecticides. Similarly, the level of resistance conferred can vary from low to very high and may differ from compound to compound. Metabolic resistance mechanisms have been identified in mosquito populations for all major classes of insecticides currently used for vector control, including organochlorine, organophosphates, carbamates, and pyrethroids.

*Esterase*s. One of the most common metabolic resistance mechanisms is that of elevated levels, or activity, of esterase enzymes which hydrolyze ester bonds or sequester insecticides. A striking example comes from studies on *Culex quinquefasciatus* that resist to a broad range of OP insecticides. In this species, multiple copies of EST-genes was already found hence enabling it to overproduce this type of enzyme [55]. In contrast to the situation in *Culex*, a number of *Anopheles* species (ie *An. culicifacies*, *An. stephensi* and *An. arabiensis*) have a non-elevated esterase mechanism that confers resistance specifically to malathion through increased rates of metabolism. Malathion resistance in *Anopheles* spp was associated with an altered form of esterase that specifically metabolizes the molecule at a much faster rate than that in susceptible counterparts [56, 57]. Although Carboxylesterase (CCEs) have been mostly associated to organophosphate resistance in mosquitoes, their role in pyrethroid resistance is probable. Indeed, the ability of esterases to metabolize pyrethroids has been suggested in mosquitoes [58, 59] even if no specific mosquito CCE has yet been validated as a pyrethroid metabolizer

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[50]. Clearly much more information is needed on the esterase-mediated resistance in malaria vectors.

P450s. Cytochrome P450-dependent monooxygenases are an important and diverse family of enzymes involved in the metabolism of numerous endogenous and exogenous compounds. P450 belong to six families and increased transcription of genes belonging to the CYP4, CYP6, and CYP9 has been observed in various insecticide-resistant species from different taxa [60]. There is increasing number of reports demonstrating elevated P450 monooxygenase activities in insecticide-resistant mosquitoes, frequently in conjunction with altered activities of other enzymes. In most cases where a link between insecticide resistance and elevated P450 activity has been shown, the CYP gene belongs to the CYP6 family. Since the publication of the *An. gambiae* genome [61], P450s were extensively studied in the primary malaria vector in Africa. A total of 111 P450 enzymes were identified [62] and, as in other insects, only a small number of these enzymes are capable of detoxifying insecticides. However, higher activity of enzymes and/or expression of detoxification genes in insecticide resistant colonies do not necessarily correlate with insecticide resistance. For example, some authors have shown elevated transcript levels of an adult-specific CYP6 P450 gene, CYP6Z1, in pyrethroid-resistant strain *of An. gambiae* [63, 64] and *An. funestus* [65]. Further validation studies conducted in *An. gambiae* showed that cyp6z1 was however not capable to metabolize pyrethroids but was capable to metabolize DDT [66]. Another study showed that CYP6z2 displays broad substrate specificity, which may be associated with xenobiotics metabolism and detoxification [67]. Despite, CYP6Z2 being able to bind to permethrin and cypermethrin, this gene does not metabolise any of these insecticides. Microarray-based approaches have lately identified three new "candidate" P450 genes that were found to be repeatedly over-produced in pyrethroid resistant populations of *An. gambiae*: CYP6M2, CYP6P3 and CYP6Z2 [68-70]. All of these genes encode for enzymes that are able to bind to type I and type II pyrethroids but only CYP6P3 and CYP6M2 showed to metabolize the insecticides [10, 71]. More recently, some authors demonstrated that CYP6M2 is also capable of metabolizing the organochlorine insecticide DDT in *An. gambiae*, hence demonstrating the first evidence for a metabolic cross-resistance in malaria vectors [9]. Interestingly the putative ortholog of *An. gambiae* CYP6P3, CYP6P9, as being the prime candidate for conferring pyrethroid resistance, have been identified in *An. funestus* [72, 73] but only the CYP6P9 showed to metabolize types I and II pyrethroids [74]. Recent works showed that overproduction of CYP6P9 in *An. funestus* result from gene duplication [72]. In *An. minimus* mosquito, CYP6AA3 and CYP6P7 were up-regulated in pyrethroid-resistance population of Thailand [75] and seem to possess activities toward pyrethroid degradation [76, 77].

Glutathione S-transferases (GSTs). Glutathione transferases (GSTs) are multifunctional enzymes involved in the detoxification of many endogenous and xenobiotic compounds. Conjugation of Glutathione (GSH) to such organic molecules enhances solubility, thus facilitating their eventual elimination [78]. Elevated GST activity has been implicated in resistance to at least four classes of insecticides in insects. Higher enzyme activity is usually due to an increase in the amount of one or more GST enzymes, either as a result of gene amplification or more commonly through increases in transcriptional rate, rather than

qualitative changes in individual enzymes [52]. At least six classes of insect GSTs have been identified in *An. gambiae* [79], found in several large clusters on all three chromosomes. The Delta and Epsilon classes found exclusively in insects are the largest classes of insect GSTs. Members of both classes have been implicated in resistance to all the major classes of insecticide. The primary role of GSTs in mosquito insecticide resistance is in the metabolism of DDT to DDE (non toxic products), although they also have a secondary role in organophosphate resistance [80]. GST-based DDT resistance is common in a number of anopheline species including *An. gambiae* [81-83], reflecting the heavy use of this insecticide for malaria control over several decades. Molecular biology and *in vitro* expression studies showed that *aggst3-2* was over expressed in resistant strain of *An. gambiae* and that recombinant *aggst3-2* was very efficient at metabolizing DDT [84]. Most studies of GSTs suggested that regulation occurs at the transcriptional level. Several regulatory elements have been identified in the promoter regions of GSTs that may mediate their induction but the significance of these findings is unclear. Genetic mapping of the major genes controlling GST-based DDT resistance in *An. gambiae* provided however evidence for a trans-acting regulator [84], although in this species, mutations in promoter elements of the Epsilon GST cluster are also associated with resistance [81]. It has been suggested that GSTs may play a role in pyrethroid resistance by detoxifying lipid peroxidation products induced by pyrethroids and/or by protecting from insecticide exposure induced oxidative stress [85]. Furthermore, GST might confer secondary role in pyrethroid resistance by sequestering the insecticide hence reducing the total *in vivo* concentration of insecticide [86].

Despite the great advance obtained recently in the identification of the role of detoxifying enzymes in insecticide resistance, force is to note that the function of >90% of metabolic genes is still unknown. Although only a limited number of resistance mechanisms have been implicated to date, the diversity within enzyme families involved in metabolic resistance is likely to contribute substantially to resistance to many insecticide classes. Further functional genomics and post-genomic technology are needed to reveal the contributions of hitherto unsuspected enzymes in insecticide metabolism and/or sequestration and to identify the causal mutations associated with metabolic resistance in mosquitoes. The contribution that these enzymes make towards various insecticide resistance phenotypes in malaria vectors is yet to be elucidated.

4.2. Target-site resistance

The second most common resistance mechanism encountered in insects is target-site resistance. Insecticides generally act at a specific site within the insect, typically within the nervous system (e.g. OP, carbamate, DDT and pyrethroid insecticides). The site of action can be modified in resistant strains of insects such that the insecticide no longer binds effectively. Reduce sensitivity of the target receptors to insecticide results from non-silent point mutations in the gene encoding the protein. For example, the target site for OP and carbamate insecticides is acetylcholinesterase (AChE) in the nerve cell synapses. Several mutations in the gene encoding for an acetylcholinesterase have been found in insects" [87] which result in reduced sensitivity to inhibition of the enzyme by these insecticides [88, 89]. In malaria vectors, the

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G119S mutation (i.e. glycine to serine substitution at position 119) responsible for carbamate and OP resistance has been reported in *An. gambiae* and *An. albimanus*, essentially at the heterozygous state [90]. Recent sequence analysis of some resistant mosquitoes collected in Benin revealed the presence of a duplication of the *ace-1* gene in both A*n*. *A. gambiae* M and S forms [91]. In addition, mutations at a single codon (position 302) in the Rdl (resistance to dieldrin) gene encoding one receptor subunit, from an alanine residue to a serine (or more rarely to a glycine), have been documented in dieldrin-resistant insect species [92] including the malaria vectors *An. stephensi* [93], *An. gambiae s.l.* [94] and *An. funestus* [31]. Similarly, mutations in the amino acid sequence in the voltage-gated sodium channels of nerve cell membranes leads to a reduction in the sensitivity of the channels to the binding of DDT and pyrethroid insecticides [95]. Alterations in the target site that cause resistance to insecticides are often referred to as knockdown resistance (*kdr*) in reference to the ability of insects with these alleles to withstand prolonged exposure to insecticides without being 'knocked-down" [96]. One of the most common amino acid replacements associated with pyrethroid resistance in malaria vectors is a substitution of the leucine residue found at codon 1014 with either phenylalanine (1014F) [97] or serine (1014S) [98] in the Voltage-Gated Sodium Channel (VGSC). Interestingly, residue 1014 does not appear to interact directly with the insecticide but is predicted to alter channel activation kinetics [99]. Note that a *de novo* mutation (N1575Y) recently emerged within domains III-IV of voltage gate sodium channel in pyrethroid resistant populations of *An. gambiae* and seems to occurs only in a single long-range haplotype, also bearing 1014F allele [100]. It has been suggested that the N1575Y mutation may compensates for deleterious fitness effects of 1014F and/or confers additional resistance to pyrethroid insecticides.

4.3. Reduced penetration

Modifications in the insect cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides can be found is some resistant insects. This resistance mechanism is not specific and can affect a broad range of insecticides. Reduced uptake of insecticide, often referred to as cuticular resistance, is frequently described as a minor resistance mechanism. Certainly for pests where the major route of insecticide delivery is via ingestion, this is likely to be the case. However, for malaria control, where insecticides are typically delivered on bed nets or on wall surfaces, uptake of insecticides is primarily through the appendages. An increase in the thickness of the tarsal cuticle, or a reduction in its permeability to lipophilic insecticides, could have a major impact on the bioavailability of an insecticide *in vivo*. Examples of reduced-penetration mechanisms are however limited; cuticular resistance was reported for the domestic Fly *Musca domestica* [101] and the lymphatic filariasis vector *Culex quinquefasciatus* [102]. In *Anopheles*, microarrays studies have recently identified two genes, cplcg3 and cplcg4, encoding cuticular proteins that were upregulated in pyrethroid resistant strains from four populations and two different species (i.e. *An. gambiae* and *An. stephensi*) ([69, 103, 104]. Recently, measures of mean cuticle thickness in a laboratory strain of *An. funestus* using scanning electron microscopy (SEM) showed that the mean cuticle thickness was significantly greater in pyrethroid tolerant mosquitoes than their susceptible counterparts [105]. Clearly

much more work is required in order to identify the significance of cuticular resistance in phenotypic resistance.

4.4. Behavioural resistance

Insecticide resistance in mosquitoes is not always based on biochemical mechanisms such as metabolic detoxification or target site mutations, but may also be conferred by behavioural changes in response to prolonged exposure to an insecticide. Behavioural resistance does not have the same "importance" as physiological resistance but may be considered to be a contributing factor, leading to the avoidance of lethal doses of an insecticide [106, 107]. For example, the first study on the irritant effect of DDT residual deposits was conducted using *Anopheles quadrimaculatus* where females were found to be irritated shortly after making contact with the treated surfaces resulting in a rapid escape response from a treated house prior to taking a blood meal [108]. This type of response can be further divided into direct contact excitation (sometimes referred to as 'irritancy') and non-contact spatial repellency that is used when insects move away from the insecticide-treated area before making direct contact [106, 109]. Examples of behavioral resistance or avoidance are few. Change in vector composition (i.e. switch from *An. minimus* to *An. harrisoni*) has been observed following implementation of ITNs in a village form central Vietnam [110]. With regard to *An. funestus*, recent findings showed a shift from indoor to outdoor biting preferences in Tanzania in relation to increasing coverage of pyrethroid-impregnated net [111]. Significant changes in the host-seeking behavior of the *An. funestus* population was confirmed in Benin (West Africa) where scaling up of LLINs at community level induced a change from night biting to early-morning biting behaviour [112]. It is unclear however whether adaptation of malaria vectors species to insecticidal based vector control interventions such as LLIN may result from a phenotypic plasticity or from selected behavioral traits (see Durnez & Coosemans for details).

5. Method to detect insecticide resistance

Currently most resistance monitoring is dependent on bioassays, using fixed insecticide concentrations and exposure times, and the data is reported as percentage mortality and/or Knock Down (KD) effect. The World Health Organisation (WHO) has defined diagnostic doses (i.e. twice the dosage that killed 100% susceptible mosquitoes of a given species) for most insecticides used in malaria control and produces susceptibility test kits consisting of exposure chambers and insecticide treated filter papers [113-115]. Although simple to perform, these diagnostic dose assays provide limited information and several alternative methods for detecting resistance are available (Table 2). These alternative assays generally detect specific resistance mechanisms, and should always be performed as an addition, not a substitute, to bioassays, to avoid the risk that unknown resistance mechanisms go undetected. It should be noted that none of the current methods listed in Table 2 are suitable for detecting cuticular and/or behavioural resistance. Regular monitoring for insecticide resistance is essential in order to react proactively to prevent insecticide resistance from compromising control. If the frequency of resistance alleles is going to build up unchecked, resistance may eventually

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become 'fixed' in the populations. Once resistance reaches very high levels, strategies to restore susceptibility are unlikely to be effective.

Method	Advantages	Disadvantages
Bioassays using WHO defined diagnostic doses of insecticide	Standardized, simple to perform, detect resistance regardless of mechanism	Lack sensitivity and provide no information about level and type of resistance (except when using with synergists), to be done on live mosquitoes
Dose response bioassays	Provides data on level of resistance in population, regardless of mechanism	Require large numbers of alive mosquitoes, and data from different groups not readily comparable
Biochemical assays to detect activity of enzymes associated with insecticide resistance	Provides information on specific mechanisms responsible for resistance	Requires cold chain. Not available for all resistance mechanisms, sensitivity and specificity issues for some assays (e.g. GST)
Molecular assays to detect resistant alleles	Very sensitive. Can detect recessive alleles and therefore provide an 'early warning' of future resistance.	Requires specialized and costly equipment. Only available for a limited number of resistance mechanisms.

Table 2. Methods for detecting insecticide resistance (source: see [6])

5.1. Bioassays

Guidelines for test procedures and interpretation of results are available from the WHOPES[3] (see http://www.who.int/whopes/resistance/en/). It is important that the mosquitoes used for the bioassays are standardized for age, sex and physiological status as all of these can affect the outcome of the tests. Typically either adults raised from isofemale lines or F1 progeny from field collected blood fed females are used. The limitations and advantages of these two alternatives have recently been discussed [116].

These diagnostic dose assays are simple to perform and provide standardized data sets that, assuming the guidelines are followed, can be readily compared to identify temporal and/or geographical variations in the resistant status of malaria vector populations. However, it is important to recognize some of the limitations of these susceptibility tests. As only a single concentration of insecticide is used, the results do not provide any information about the level of resistance in a population. For example if 50 % of population A and 20 % of population B were killed after exposure to the diagnostic dose of permethrin, it cannot be concluded that population B is more resistant than population A. The results only indicate that both populations are resistant (according to WHO definitions if there is < 80 % mortality, the population is defined as resistant) and that, subject to tests of significance, there is a higher frequency of

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resistant individuals in population B than in A. Dose response assays would be needed to compare the levels of resistance in two populations (e.g. by measuring the Resistant Ratios and their 95% confidence intervals). For pyrethroids, median knock down time (MKDT) is also a useful quantifiable variable [117]. Similarly, the results of these tests cannot be used to compare the levels of resistance to two different insecticides. If 50 % mortality was observed after exposure to the diagnostic dose of permethrin (0.75 %) whereas mortality was 70% after exposure to the diagnostic dose of deltamethrin (0.05%), it is not correct to state that the population is more resistant to permethrin than deltamethrin. Again, all that can be stated is that the population is resistant to both insecticides.

Partly due to the limitations of the diagnostic dose assays described above and partly due to the difficulties that are sometimes incurred in obtaining a regular supply of the insecticide impregnated papers from WHO, an alternative bioassay methodology has been developed [118] and is being adopted by some monitoring programmes. This method, known as the CDC bottle bioassay, uses glass bottles coated with a known concentration of insecticide. As these test kits are assembled in the users own laboratory, the concentration of insecticide can be readily adjusted enabling dose response curves to be developed to compare two or more strains. A caveat to this is that the flexibility, and the potential variation in the insecticide grade used in the tests, impairs comparison of results between two separate studies.

Both WHO diagnostic doses and CDC bottle bioassays can be modified to incorporate synergists. Synergists such as piperonyl butoxide, that block the activity of two major detoxification enzyme families, can be used to explore the role of different resistance mechanisms. If resistance is due to increased metabolism, exposure to an appropriate synergist prior to insecticide bioassays should increase the level of mortality observed.

5.2. Biochemical tests

Biochemical tests to detect alterations in activities of enzyme families associated with insecticide resistance have been available for over two decades and are sometimes used in combination with insecticide bioassays [119]. These assays employ model substrates to record the overall activity of glutathione transferases, carboxylesterases or cytochrome P450s in individual insects. Biochemical assays are also available to detect target site resistance to organophosphate and carbamate insecticides caused by insensitive acetylcholinesterase (AChE). The enzymatic reaction produces a colour change that is generally visible to the naked eye and hence these assays do not require access to expensive equipment (spectrophotometer is appropriate). However, it is important that the mosquitoes are kept on ice from the point of collection to the performance of the assay and this can often pose logistical challenges. Furthermore, there are sensitivity and specificity issues that limit the utility of some of these assays. For example, with over 100 different cytochrome P450 enzymes in malaria vectors, an assay that measures the total level or activity of this enzyme family may not have the sensitivity to detect over production of the single or small number of P450 enzymes that are thought to be involved in pyrethroid metabolism. This may explain the lack of significant correlation observed in many studies between cytochrome P450 activity and bioassay mortality results [120, 121]. In addition not all members of the enzyme family will have the same affinity for the model substrates used in these assays (e.g. CDNB (1-chloro 2-4, dinitrobenzene) is the substrate

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typically used to assess glutathione transferase activity but the Epsilon class of GSTs which are responsible for DDT resistance have relatively low activity with this substrate). In order to incorporate data from resistance monitoring into evidence based decisions on appropriate insecticide based interventions for malaria control, it is clearly essential that the data is both reliable and accessible. Although guidelines for conducting the various assays exist, there is little consensus on the number of sites and frequency with which resistance monitoring should occur [122]. It is clear that resistance is a dynamic trait, and wide fluctuations in resistance levels throughout the malaria transmission season have been reported [116, 123, 124]. Resistance can also be very focal, particularly when vector composition differs between sites [125], hence a minimum number of sampling sites should be established, taking into account patterns of vector distribution and insecticide usage. The WHO/AFRO African Network for Vector Resistance was established in 2000 and amongst its objectives was the important goal of improving the dissemination of resistance data. Accordingly a database was established to store the results of resistance monitoring activities by the African Network for Vector Resistance (ANVR) members but until recently, this database was not readily accessible by outside users. The recent establishment of new data base (see section 6), as an online centralized resource for collating data on insecticide resistance in disease vectors and the integration of this with the ANVR database, will hopefully ensure that both published and unpublished data on resistance in malaria vectors are more readily available to all interested parties.

5.3. Molecular tests

A multitude of molecular assays have been developed to detect *kdr* alleles in malaria mosquitoes, several of which were recently compared in a study by Bass et al (see [126]). These are routinely used by research laboratories monitoring for insecticide resistance and are gradually being incorporated into some national malaria control resistance monitoring programmes. Unfortunately, despite the recent identification of the key enzymes responsible for metabolic resistance to pyrethroids in *An gambiae* and *An funestus*, there are currently no simple DNA based assays to detect these resistance mechanisms. Detection of these genes is presently dependent on RNA based approaches using relatively sophisticated equipment (e.g. RT-qPCR). Assays to detect the genetic mutation(s) responsible for the resistance phenotype in individual insects can provide an early warning of the emergence of resistance which may not have been detectable by bioassays that can only record the population response. The presence of a single individual with an allele known to confer resistance should be cause for concern as experience dictates that resistance can spread very rapidly in a population unless the selection pressure is eased and/or the genetic cost associated with the resistant allele is high. Conversely, a negative result from a molecular assay should not lead to complacency. As discussed above, molecular assays are presently only available for target site resistance and the failure to detect *kdr* clearly cannot be interpreted as an absence of resistance in a population. Hence molecular assays should be seen as a complement rather than a substitute for bioassays.

6. Current distribution of insecticide resistance

Insecticide resistance has been reported in the main malaria vectors worldwide. Resistance is however not uniformly distributed among vector species and can greatly differ from one village, province, country, region and continent to another. Unfortunately, the highest levels of insecticide resistance were reported in Africa where malaria burden is still the highest in the world [1]. Resistance to pyrethroids, the gold standard insecticides used for LLIN and IRS will be extensively discussed in the present chapter as it remains a real and ever-present danger to future success of malaria vector control. Note that more information on the distribution of insecticide resistance in malaria vectors can be found in *Anobase*, http://anobase.vectorbase.org/ir/; *MARA* http://www.mara.org.za; *Arthropod Pesticide Resistance Database*, http://www.pesticideresistance.org.; and IR mapper, http://www.irmapper.com.

6.1. Africa

Although the occurrence of insecticide resistance in malaria vectors in Africa is not a "new" event (see section 2.), the speed at which pyrethroid-resistance recently evolved in field populations is worrying as it may jeopardize the current malaria vector control initiatives carried out in the continent. As shown in figure 2, pyrethroid resistance in *Anopheles* sp. is widespread but not uniformly distributed among the different countries. In the 49 African countries that have been investigated (see [6] for details), 15 did not report any data on resistance in the last 10 years i.e. Algeria, Botswana, Democratic Republic of Congo, Djibouti, Sierra Leone, Lesotho, Liberia, Libya, Maurice, Mauritania, Namibia, Rwanda, Somalia, Swaziland, Tunisia. If a lot of data has been generated in West Africa (as far as *An. gambiae s.l.* is concerned), a lack of information is globally observed in Central, Eastern and Austral Africa. It is obvious that the frequent conflicts that has occurred in the last decades in some African countries has rendered difficult the conduct of routine monitoring surveys by NMCP, International Organisation (WHO/ANVR) and/or research institutions.

Globally, pyrethroid resistance is high in *An. gambiae s.l.* in West Africa including Benin [127], Burkina Faso [128], Guinea Konakry ([129], Ghana [130], Mali [131], Niger [132], Nigeria [133] and Cote d'Ivoire [134]). In this region, pyrethroid resistance is predominant in *An. gambiae s.s.*, compared to *An. arabiensis*. Surprisingly, susceptibility to pyrethroids (permethrin and/or deltamethrin) was reported in *An. gambiae* s.l. in Guinea Bissau [135] despite the presence of the L1014F mutation. In Central Africa, pyrethroid resistance/tolerance is widespread in *An. gambiae s.l* in Cameroon [136-138], Chad [116, 139], Gabon [140, 141], Equatorial Guinea [8] and Sudan [142, 143]. In Chad, North Cameroon and Sudan, pyrethroid resistance is present essentially in *An. arabiensis,* which is consistent with the higher prevalence of this mosquito species in more arid areas with higher mean annual temperatures [144]. In East and Austral Africa, *An. gambiae* and *An. arabiensis* populations are mostly susceptible to pyrethroids in Tanzania [145, 146], Mozambique [147] and Madagascar [148], but highly resistant in eastern Uganda [149, 150], Ethiopia [151], Kenya [152, 153], Zambia [154], South Africa [155] and the Gwave Region of Zimbabwe [120]. Regarding *An. funestus*, most of the literature reporting pyrethroid resistance comes from South Africa [39, 156] and Mozambique [157-159], most probably because *An. funestus* is the main malaria vector in these countries. The data available

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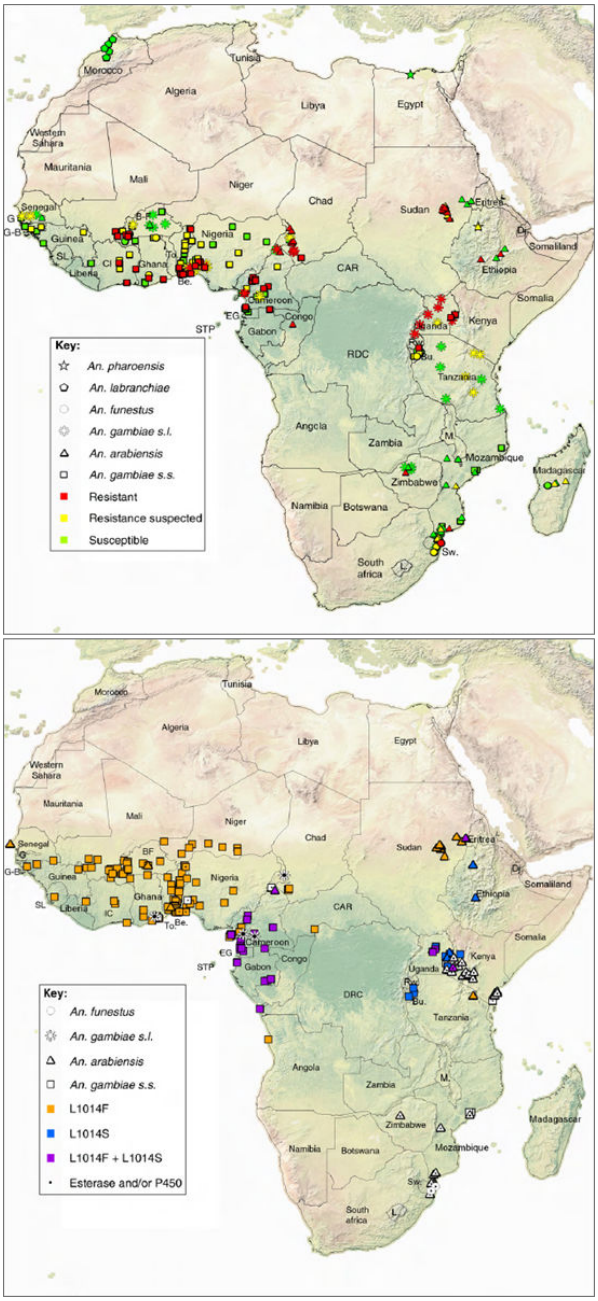

Figure 2. Maps showing the distribution of pyrethroid-resistance in African malaria vectors; A) status of pyrethroid resistance according to WHO criteria ; B) Target site (*kdr*) and metabolic resistance reported for a given mosquito species (Source; see [6]).

in other African countries is very limited in partly due to the difficulty to colonize *An. funestus* species in laboratory. Pyrethroid resistance/tolerance was detected in Malawi [160, 161] and suspected in Obusi and Kassena-Nankana Regions from Ghana [121, 162] and Benin [163] whereas full susceptibility to permethrin and deltamethrin was found in Burkina Faso [164] and Tanzania [145]. There is a lack of information on secondary vectors e.g. *An. moucheti* and *An. nili* which can play important role in malaria transmission in specific settings (e.g. Cameroon, Congo, Côte d'Ivoire). Regarding other vectors species, full susceptibility to pyrethroids has been reported in *An. labranchiae* in Morocco [165] and in *An. pharoensis* in Egypt [166] and Ethiopia [167].

In Africa, the L1014F mutation is widespread (figure 2) and predominant in the molecular S form compared to the M form, except in Benin [168], Guinea Equatorial [8] and Niger [132]. Some authors suggested that the *kdr* alleles may have arisen from at least four independent mutation events in the *An. gambiae* S-form [169]. Regarding the M form, it is not clear whether the *kdr* mutation resulted from an introgression from the S form only [170, 171] and/or from independent mutation events, has recently suggested for Bioko Island [172]. The second mutation, a leucine–serine substitution at the same codon (L1014S), was identified first in a colony of *An. gambiae s.l.* from Kenya [98]. This substitution has been lately reported in Burundi [173], Cameroon [136, 138], Gabon [174], Equatorial Guinea [175], Uganda [176], Republic of Congo [177] and Angola [140], mainly in co-occurrence with the 1014F *kdr* allele. Although some authors have reported that the 1014S allele may confer lower level of pyrethroid resistance than the 1014F allele [178], its spread from eastern to central Africa and more recently to West Africa [124, 179] suggest a survival advantage of mosquitoes sharing this mutation in presence of pyrethroids. So far, the L1014S substitution has always been detected in the S molecular form [180] but recent findings showed the occurrence of the 1014S allele in the M form in Equatorial Guinea [181] and Cameroon [182]. In these two countries, the 1014S allele was present at very low frequencies, alone or associated with the L1014F allele. It is currently impossible to know whether the *kdr* alleles have arisen first in Cameroon or Equatorial Guinea. The higher frequency of the 1014S allele in the S form compared with the M form could either be attributed to an introgression from the S taxon or to a *de novo* mutation. Regarding the sister taxa *An. arabiensis*, both of these mutations were reported in Western [124], Central [183] and Eastern Africa [184]. Interestingly, a new *kdr* mutation (N1575Y) occurring within domains III-IV of voltage gate sodium channel was found in both S and M molecular forms of *An. gambiae* and occurs upon a 1014F haplotypic background only [100]. Additive resistance of *1575Y* was demonstrated for permethrin and DDT in both molecular forms of *An. gambiae*. The prevalence of the 1575Y mutation has increased in West Africa in the last years hence indicating that the 1014F-1575Y haplotype is under strong selection pressure (Djégbé pers. com). It is possible that besides the 1014F/1014S *kdr* mutation, other mutations in the para-type sodium channel gene might be needed for mosquitoes to survive after exposure to a discriminating concentration of an insecticide. Further investigation is needed to better address the distribution and the role of the N1575Y mutation in pyrethroid resistance as well as to assess the fitness benefits conferred by this allele on the L1014F mutation in malaria vectors.

Beyond the spread of *kdr* alleles, metabolic-based resistance due to detoxifying enzymes namely oxidase, the GST (epsilon) and CCE families have expanded in African malaria vectors.

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In *An. gambiae s.l.* metabolic resistance involving increased levels of P450 has been reported at least in Kenya [185], Cameroon [186], Benin [69], Nigeria [69], Ghana [70], Mozambique [147], South Africa [187] and Zimbabwe [120]. Up to now, only genes encoding CYP6P3 and CYP6M2 P450 enzymes have been clearly involved in cellular mechanisms known to metabolize deltamethrin and permethrin [10, 71].These genes were found over-expressed in pyrethroid-resistant *An. gambiae* populations from Benin, Nigeria and Ghana [69, 70], mainly in co-association with the *kdr* L1014F allele. In *An. funestus*, pyrethroid resistance involving increased activity of P450 monooxygenase and/or GST was demonstrated in South Africa [157], Mozambique [188] and Malawi [161].

To conclude, the immense challenge in Africa will be not to manage and control *kdr*-resistant mosquitoes only but to deal with the development of "multiple resistant" populations that could resist to different class of insecticides used in public health. One other issue is the occurrence and development of carbamate resistance in some countries (eg Benin, Nigeria) where this chemical class is in use for IRS through the PMI programme [47, 48]. The spread of carbamate resistance in malaria vectors in Africa is worrying for insecticide resistance management and alternative insecticides, and innovative strategies are urgently needed to better reduce the vectorial capacity of mosquitoes and hence effectively reduce the burden of malaria in the region. Resistance management strategy for malaria control is discussed in section 8.

6.2. South-East Asia and India

The South East Asia Region (SEAR) that account for 13% of the total malaria cases worldwide (2nd position after Africa) [1] is not spare of insecticide resistance in the main malaria vector species.

In the Mekong region, cross-country monitoring of insecticide resistance has been conducted through the MALVECASIA network (http://www.itg.be/malvecasia/) to help MCPs in the choice of insecticide to use at regional level. Large differences in insecticide resistance status were observed among species and countries. *Anopheles dirus s.s.*, the main vector in forested malaria foci, was mainly susceptible to permethrin except in central Vietnam where it showed possible resistance to type II pyrethroids [23]. *Anopheles minimus* s.l. populations were found resistant / tolerant in Vietnam and northern Thailand [189] but almost susceptible in Cambodia and Laos. No *kdr* mutation has been observed so far in these species [190] and pyrethroid resistance seems to result from increased detoxification by esterases and/or P450 monooxygenases [191]. Indeed, increased mRNA expression of two P450 genes, *CYP6P7* and *CYP6AA3*, suspected to metabolize some pyrethroids [76] have been reported in a deltamethrin-resistant population of *An. minimus* in Thailand [75, 192].

Anopheles epiroticus of the Sundaicus Complex showed to be highly resistant to all pyrethroids in the Mekong Delta [23] but susceptible to DDT, except near Ho Chi Minh City. DDT and pyrethroid-resistant populations of *An. subpictus* were reported in Vietnam and Cambodia. Biochemical assays suggest an esterase-mediated pyrethroid detoxification in both *An. epiroticus* and *An. subpictus* whereas DDT resistance in *An. subpictus* might be conferred to a higher GST activity. In Vietnam and Cambodia, *An. vagus* and *An sinensis* showed various

levels of pyrethroid resistance and sequence-analysis of the DIIS6 region of the VGSC revealed the presence of the 1014S *kdr* allele [193]. Pyrethroid resistant populations of *An. sinensis* were also reported in the Republic of Korea (ROK) [194] and in China [195]. In China, cypermethrin resistance in *An. sinensis* was associated with the presence of both 1014F and L1014C substitutions, whereas only 1014F and 1014S mutations were found in the ROK and Vietnam, respectively. In Indonesia, molecular analysis carried out in field mosquito samples revealed the presence of the 1014F allele in the four main malaria vectors i.e. *An. sundaicus*, *An. aconitus*, *An. subpictus* and *An. vagus* [196]. At the present time, it is difficult to speculate on the relative contribution of the *kdr* mutations *versus* metabolic detoxification on pyrethroid and DDT resistance in malaria vectors from the SEA region and more work are needed to establish a clear trend.

Insecticide resistance is known to be widespread in other part of Asia such as India. In this country, resistance has a long history (see section 2) and it represents a big challenge for malaria vector control. Among the *Anopheles* species, *An. culicifacies s.l.*, the major vector of malaria in most parts of the country, has developed strong resistance to pyrethroids [36], DDT [197, 198], dieldrin/HCH [199], and malathion [198]. The 1014F mutation, which generates the *kdr* phenotype was detected in pyrethroid and DDT resistant *An. culicifacies s.l.* populations sometimes in co-occurrence with the 1014S mutation [197]. Note that a novel mutation V1010L (resulting from G-to-T or -C transversions) in the VGSC was recently identified in Indian *An. culicifacies* and was tightly linked to 1014S substitution [197]. Elevated activities of GST seem to play also an important role in DDT-resistance in this mosquito species [82]. Similarly, strong level of pyrethroid resistance due to the presence of both 1014F and 1014S mutations was found in the urban malaria vector *An. stephensi* particularly in the Rajasthan District [200]. Other vectors that are reported to be resistant to pyrethroid, DDT and/or dieldrin/HCH in India are *An. annularis*, *An. subpictus* and *An. philippinensis* [201]. In contrast, *An. minimus* has still not showed pyrethroid and DDT resistance [202].

The same trend was noted in Sri Lanka where the main malaria vectors species, i.e. *An. culificifacies s.l.* and *An. subpictus* have developed DDT, pyrethroid and malathion resistance in several districts [203, 204]. However, the main mechanisms associated with DDT and malathion resistance in *An. culicifacies s.l.* and *An. subpictus* are primarily metabolic and involve carboxylesterases (malathion) or monooxygenases and GSTs (for DDT) [205, 206]. An altered acetylcholinesterase conferring organophosphate resistance has been suspected in both vector species [205].

In the delta region of Bangladesh, the *An. sundaicus* malaria vector is fully susceptible to DDT but other malaria vectors such as *An. philippinensis*, *An. maculatus s.l.*, and *An. aconitus* have all developed resistance to DDT [207]. *Anopheles aconitus,* additionally, has been reported to be resistant to dieldrin/HCH. Bhutan records *An. maculatus s.l.* as resistant to DDT, but there is no record of its resistance to any other insecticides [207]. Two vectors of malaria in Nepal, *An. maculatus s.l.* and *An. aconitus*, also have developed resistance to DDT whereas only malathion resistance was reported in *An. stephensi* in Pakistan [208]. Finally, in Iran and Turkey, *An. stephensi* and *An. sacharovi* showed resistance to DDT and dieldrin but both species are mostly susceptible to pyrethroids [209-211].

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6.3. Latin America

The countries of the Amazon Basin (Bolivia, Brazil, Colombia, Ecuador, Guyana, Peru, Surinam and Venezuela) carry the greatest burden of malaria in the Americas. The primary vectors of this disease in the Amazon basin are *An. darlingi* and *An. albimanus*. Surprisingly, much less data on insecticide resistance is available for these two mosquito species comparatively to African and/or Asian malaria vector species [212].

In Colombia, DDT resistance was reported in the late 80's in some populations of *An. darlingi* in the districts of Quibdó and close to the Atrato River [213, 214]. Successive insecticide susceptibility evaluations revealed resistance to pyrethroids in both *An. darlingi* and *An. albimanus* mainly in the Chocó State [215]. In *An. darlingi*, increased levels of both Multi function Oxidase (MFO) and Non specific Esterase (NSE) were reported in a deltamethrin and DDT-resistant population, hence suggesting a possible involvement of these detoxifying enzymes in cross resistance to DDT and deltamethrin [35]. Note that various levels of resistance to organophosphate and pyrethroids were also reported in the secondary malaria vector *An. nuneztovari* [216].

In neighboring countries, DDT, permethrin and deltamethrin resistance was found in laboratory colonized populations of *An. albimanus* from Guatemala, whereas full susceptibility was noted in field populations from El Salvador and Belize [217, 218]. The colonies from Guatemala showed significant increase in the specific activity of esterase and/or oxidase as measured by spectrophotometer suggesting their potential involvement in pyrethroid-resistance [34, 217]. In Peru, monitoring campaigns carried out since 2000 showed that *An. albimanus* was the only Anopheline species to exhibit pyrethroid-resistance [219].

In Mexico, high level of DDT resistance and low levels of resistance to organophosphate, carbamate and pyrethroid insecticides were detected in field populations of *An. albimanus* in Chiapas, prior to a large-scale resistance management project [220]. Biochemical assays revealed that the DDT resistance was caused by elevated levels of GST activity leading to increased rates of metabolism of DDT to DDE [22], whereas carbamate resistance was attributed to an altered acetylcholinesterase (AChE). More recent studies conducted in the southern Yucatan Peninsula showed high levels of DDT, deltamethrin and pirimiphos-methyl resistance in the *An. albimanus* populations tested [221]. Biochemical tests revealed elevated levels of GST, P450 and esterases activities that could be involved in DDT and pyrethroid-resistance. As for carbamate, pirimiphos-methyl resistance was strongly correlated with the presence of an insensitive acetylcholinesterase.

To our knowledge, it is the main "published" information available on the distribution, levels and mechanisms of resistance (i.e. accessible through Medline and pub med) in malaria vectors in Latin America. It is then essential to strengthen the capacity of all Latin America countries that suffering from malaria to make insecticide monitoring in routine to obtain much accurate information on the insecticide resistance situation in the malaria vectors. This will provide stake holders with useful information for the implementation of more effective and sustainable malaria control programmes in the region.

7. Impact of pyrethroid-resistance on programmatic malaria control

Few operational reports exist that measure the impact of pyrethroid resistance on epidemiological outcomes of malaria, owing to body of factors that mislead the attributable component of resistance. Where tentative evidence is provided in most cases, the design of the study has been observational and the effect of confounding factors can never be excluded with confidence, making difficult the interpretation of data.

Most probably, the only clearest evidence of control failure being directly linked to pyrethroid resistance was reported from the borders of Mozambique and South Africa. In 1996, the malaria control programme in KwaZulu Natal switched from using DDT to deltamethrin for indoor spraying. Within four years, notified malaria cases had increased about four fold, *An. funestus* had re-appeared and was observable emerging alive from pyrethroid sprayed houses. Bioassays showed that this species was resistant to pyrethroids but susceptible to DDT [39]. A decision was taken to switch back to DDT spraying and, within the two years after this switch was made, *An. funestus* was no longer observed emerging alive from insecticide sprayed houses. The combination of DDT and antimalarial drugs in KwaZulu-Natal has resulted in a 91% decline in the malaria incidence rate [222, 223]. There is no doubt that that the emergence of pyrethroid resistance and the avoidance of its effects by switching to DDT, has been of major operational importance [224].

Additional evidence was brought on the island of Bioko on the West African Coast. A malaria control strategy based on IRS with lambdacyhalothrin was launched by the Bioko Island Malaria Control Project (BIMCP) funded by the Government of Equatorial Guinea and a consortium of private donors led by Marathon Oil Corporation. One round of IRS using the pyrethroid deltamethrin (K-Orthrine WP50, Bayer Crop Sciences, Isando, South Africa) failed to curtail an increase in the population density of *An. gambiae* M form because of evidence in the rise of the knock-down resistance (*kdr*) gene in this species [8]. The programme switched to carbamate insecticide before a substantial decline in the mosquito population, transmission index and malaria prevalence in children was seen. Nevertheless, in an observational study such as this, the possible contribution of other confounding factors to the failure of pyrethroid IRS cannot be overlooked so the direct consequence of the *kdr* frequency is unclear.

Another programmatic study was conducted in the highland provinces of Burundi. Between 2002 and 2005, a well targeted vector control programme (conducted in foot of valleys only) combining IRS with pyrethroids and/or PermaNet 1.0 LLINs was initiated in one of the most affected island provinces, Karuzi [225]. Initially, one round per year of pyrethroid-IRS was carried out in all human dwellings and cattle sheds before the seasonal increase in transmission. LLIN distribution preceded the first IRS round in the same year. The S-form of *An. gambiae* was the predominant vector species in Karuzi District and showed resistance to pyrethroids due to the *kdr* mutation. The entomological data showed that the intervention, overall, effectively reduced *Anopheles* density by 82% and malaria transmission was decreased by 90% despite high frequencies of the L1014S allele in the local *An. gambiae* population [173].

In a more recent observational study conducted in Malawi, the impact of pyrethroid resistance on operational malaria control has been assessed with more controversial evidence of

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resistance impacting pyrethroid-based vector control [161]. In this trial, pyrethroid-LLINs were distributed to communities in 2007 followed by a pilot campaign of IRS with lambda-cyhalothrin supported by the President's Malaria Initiatives between 2008-2010 within districts. A series of sentinel sites were established during these periods to track the effect of the increase in pyrethroid resistance in the local malaria vectors (*An. gambiae* and *An. funestus*) and assess any impact on malaria transmission and prevalence of infection. Pyrethroid resistance had been selected over the 3 years of the programme in these two major malaria vectors with the resistance in the later vector (i.e; *An. funestus*) being metabolically-mediated and involving the up-regulation of two duplicated P450s. The selection of resistance over 3 years had however not triggered a major increase in parasite prevalence in Malawian children, but it may have reduced the benefit of introducing IRS alone in several districts [161]. The impact of this pyrethroid resistance on the ability of LLIN and IRS to reduce malaria infection in Malawi needs to be further elucidated.

Similarly, in the Dielmo Village of Senegal, a longitudinal study of inhabitants was carried out between January, 2007, and December, 2010 [226]. In July, 2008, deltamethrin-LLINs were provided to all villagers and asymptomatic carriage of malaria parasites was assessed from cross-sectional surveys. Overall, the incidence density of malaria attacks decreased from 5.45 per 100 person-months before LLINs distribution in 2007 to 0.41 by August 2010, but increased sharply back to 4.57 between September and December, 2010, i.e, in less than 3 years after the distribution of LLINs. Within the same time frame, the malaria vector became gradually resistant to pyrethroids and the prevalence of the 1014F *kdr* resistance allele increased from scratch, i.e. 8% in 2007 to 48% in 2010. Once again, these results should be considered with caution as the study was conducted in an unique village and the conclusions drawn could not be extrapolated or extended to Senegal or other areas of Western Africa. Moreover, the link between the slight rise of pyrethroid resistance and the rebound in malaria cases cannot be established with accuracy and such rebound could be due to other sources of factors totally independent of resistance.

Another recent study reports the presence of pyrethroid-resistance in malaria vectors *versus* the gain in current efforts to control malaria in the Zambia [154]. In line with the Global trend to improve malaria control efforts, a country wide campaign of Olyset Nets and PermaNets (LLIN) distribution was initiated in 1999 and indoor residual spraying with DDT or pyrethroids was reintroduced in 2000 in the country by the NMCP. In 2006, these efforts were strengthened by the PMI. Both major malaria vectors, *An. gambiae* and *An. funestus* were controlled effectively with the ITN and IRS programme in Zambia, maintaining a reduced disease transmission and burden, despite the discovery of DDT and pyrethroid resistance in the country.

There have been extensive randomized controlled trials (RCTs) (phase III) in part of Africa aiming at investigating the efficacy of ITNs for malaria prevention [227], but very few have assessed how pyrethroid resistance might affect the effectiveness of such intervention. RCTs entail a set of communities randomly divided into groups, one that receives the novel form of vector control intervention, and comparison arms that often receive the old form of vector control tools or nothing. The key difficulty is that it is impossible to address the question to

whether vector control would produce a smaller reduction in malaria if the vector mosquitoes are resistant than it would have done if they were susceptible, using RCT methods. This is simply because resistance is not an easy factor that can be allocated randomly to some communities and not to others. The distribution of resistance is patchy and its severity seems to differ from one location (village) to another. Moreover there may be more resistance or survival trend of mosquitoes in some villages than others because of variations in the quality of vector control operations, or in mosquito behavior [228, 229]. This is important to mention, because many health scientists regard evidence from randomized-controlled studies as the only reliable basis for decision-making in public health.

The first RCT that investigated the impact of pyrethroid-resistance on LLIN efficacy was conducted in the Korhogo area in the north of Côte d'Ivoire. The trial encompassed multiple villages where the 1014F *kdr* allele frequency was >90% [28] and malaria was endemic. The regular use of conventionally lambdacyhalothrin-treated nets had a significant impact on the entomological inoculation rate (55% reduction) and on malaria incidence in children < 5 (56% reduction of clinical attacks) compared to a control group having no nets [230]. This was the first clear-cut evidence of ITNs continuing to provide effective personal protection against malaria in an area with a very high frequency of *kdr* in the vector population. However, as reported in Ranson et al. [6], the absence of a physical barrier in the control group may have overestimated the impact of pyrethroid treated nets against *kdr* mosquitoes in this study.

More recently, another RCT of LLINs and/or IRS was conducted in 28 villages in southern Benin, from 2007 to 2010 [231]. The objective of the study was to examine whether carbamate-IRS applied every 8 months, as practiced by the PMI programme in Benin provided additional benefit over LLINs (ie Permanet 2.0) in term of malaria prevention and management of pyrethroid resistance in malaria vectors. Results showed that combination of LLINs and IRS did not reduce malaria transmission and morbidity compared to LLIN alone in an area of pyrethroid resistance [124]. Significant increase of 1014F *kdr* frequency was observed in the reference and treated arms only 18 months post intervention hence indicating that LLIN and IRS failed to reduce the spread of the 1014F allele in malaria vectors. The authors suggested that the increase in pyrethroid resistance might have accounted for the reduction of LLIN efficacy at a community level. Clearly, further investigation is needed to assess whether pyrethroid-resistance can reduce efficiency of LLINs and IRS for malaria prevention in Africa.

Given the many obstacles for evaluating the epidemiological impact of resistance, other alternative methods to measure operational impact has been to measure proxy entomological outcomes, such as the relative mortality and feeding success of resistant and susceptible vectors in experimental huts [232, 233]. Although such results can be remarkably clear, and definitively linked to resistance, experimental hut methods have their own limitations owing to the controlled hut structures that differ in many ways to normal houses in rural African context.

An early experimental hut trial of ITNs was conducted in the western African country of Benin. In southern Benin (Ladji), pyrethroid resistance has evolved in the M form of *An. gambiae* mosquitoes that appear to combine the knockdown resistance (*kdr*) gene with oxidase mechanisms [127, 234]. In Ladji, carrier mosquitoes of this resistance were not controlled by pyrethroid treatments in experimental hut trials of ITNs or the leading brands of LLINs, PermaNet 2.0 (Vestergaard Frandsen SA, Aarhus, Denmark) and Olyset (Sumitomo Chemi-

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cals, Osaka, Japan) [235]), compared to Malanville in the north where the vector was largely susceptible to pyrethroids [127]. Further household randomized trial conducted in northern susceptible and southern resistance areas demonstrated that lambdacyalothrin-ITNs (regardless the physical condition) lose their capacity to confer personal protection against pyrethroid-resistant A*n. gambiae* [236].

One of the problems associated with many of these studies is that, due to the lack of molecular markers for alternative resistance mechanisms (i.e. metabolic or even cuticular and behavioral), the frequency of *kdr* alleles is frequently used as a proxy for resistance. It has been recently demonstrated in Southern Benin that *kdr* by itself in *An. gambiae* does not seem to bear more malaria parasites than in a susceptible [237] but this conception can be misleading when metabolic or other resistance mechanisms are predominant or combine with *kdr* to confer resistance. There is an urgent need for properly controlled large-scale trials to assess the impact of pyrethroid resistance on IRS and ITNs in Africa but also in different regions affected by malaria (e.g. Asia and Latin America). Such studies should use both entomological and epidemiological indices and should be conducted in areas where alternative resistance mechanisms are known to be responsible for pyrethroid resistance. Furthermore, these studies must consider the possibility of behavioural resistance as recently suggested in Benin [238] and Tanzania [111] and monitor for changes in key traits such as location of resting and feeding which may impact on the efficacy of current insecticide based interventions.

8. Resistance management strategies

As a general statement, the use of insecticides does not create resistance by itself but select small proportion of individuals having a genetic mutation that allow them to resist and survive the effects of the insecticide. If this advantage is maintained by constant use of the same insecticide, the resistant insects will reproduce and the genetic changes that confer resistance will be transferred to offspring so that they become more prevalent within the population (figure 3). This selection process will take longer time to occur if the gene conferring resistance is rare or present at a low prevalence. Resistance should not be confused with "induction" that can occur after sub-lethal (or low dose) exposure to any insecticide and/or xenobiotic and is not passed on to offspring [239].

8.1. Main factors influencing resistance development

The evolution of insecticide resistance is complex and depends on several genetic, biological and operational factors [240-242]. The biological factors relate by the life cycle of the insect (e.g. rate of reproduction, number of generation/offspring, rate of migration and isolation, etc), while the genetic factors include the intrinsic characteristics of the resistant genes (e.g. mono *versus* polygenic resistance, dominance, fitness cost and gene interaction). Operational factors concern the treatment itself including the method and frequency of application, dosage and residual activity of the insecticides as well as insecticide coverage.

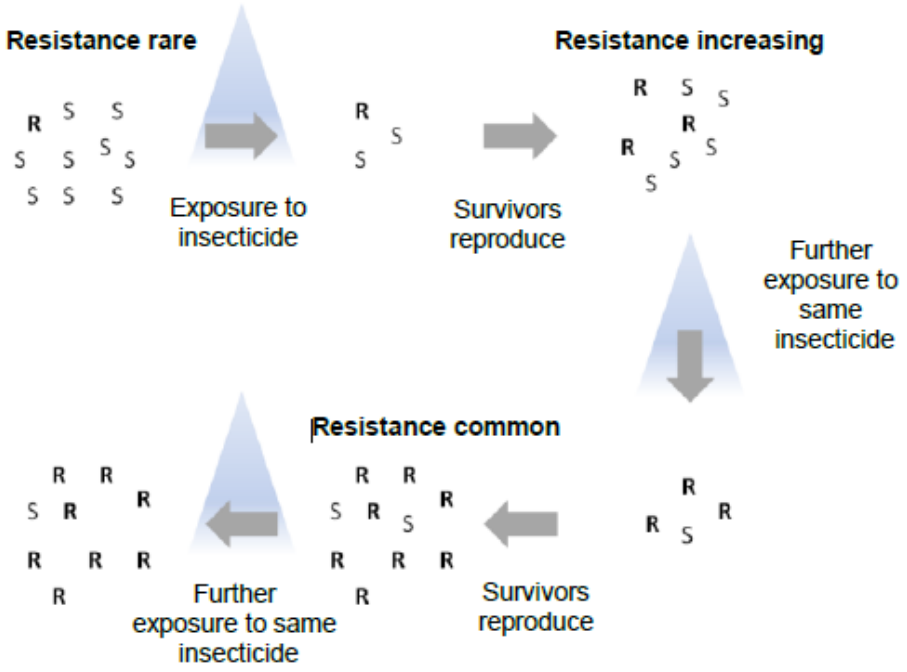

Figure 3. Possible scenario for resistance development in a mosquito population (source; [240])

8.1.1. Biological factors

Rate of reproduction

Insect species that have a short life cycle and high rates of reproduction are likely to develop resistance more rapidly than species that have a lower rate of reproduction, as any resistance genes can rapidly spread throughout the population. Because mosquitoes can produce high number of offspring (i.e. females can lay several hundred eggs during their reproductive life) they are much likely to develop resistance to insecticides than other species.

Population migration / isolation

With mosquitoes, the goal is to eliminate all or the majority of the population, however the greater the selection pressure that is put on a population, the faster susceptibility may be lost. Immigration of individuals possessing susceptible genes from untreated areas can beneficially dilute and compete with the resistance genes in the overall population. An early step in a malaria vector control programme should therefore be to estimate the susceptibility status of vector populations (see section 5 for details) and estimate potential immigration of untreated insects. This can be achieved by using genetic markers to estimate the gene flow (migrants) and genetic structure between populations. For example, an isolated area (e.g. island) where the entire area is treated would have a higher risk of developing resistance as few "susceptible"

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genotypes would join the treated population. The risk of insecticide resistance developing should be considered when planning a resistance management strategy. Awareness of and coordination with neighboring vector control programmes and agricultural activities should be encouraged, so that the regional and potential "side effect" on the target population is considered.

8.1.2. Genetics factor

Dominance

Resistance genes can range from dominant through semi-dominant to recessive. If dominant or semi-dominant, only one parent needs to possess the characteristic to be fully or partially expressed in the offspring. If recessive, both parents must possess the trait. Fortunately, most resistance mechanisms (e.g. *kdr*) are controlled by recessive or semi-recessive genes, which slows their spread within the population at early stage of resistance development when most individuals are present at heterozygous state. In contrast, when the resistance is genetically dominant, e.g. the *Ace.1*^{R} gene conferring cross-resistance to carbamates and OPs [243], it can rapidly become established within the population and will be difficult to manage. Fortunately, strong genetic cost is often associated with dominant resistant gene that can compensate the effect of the dominance and slow down the increase of resistance gene frequency in natural populations [244].

Gene interactions

Epistasis is the non-additive interaction (synergistic *versus* antagonistic) between different loci which contribute to a phenotype [245]. Epistasis between independent loci conferring insecticide resistance is important to investigate as this phenomenon can shape the rate at which resistance evolves and can dictate the level of resistance in the field. Epistasis can be measured in laboratory studies on susceptible and resistant colonies, but without these data, it is generally impossible to predict whether or not it will occur when two genes are being evaluated. Studies of the interactions between resistance loci have been most commonly conducted in house flies [246-248]. Generally, a greater than additive interaction was observed between two loci that were both homozygous resistant, whereas additively (i.e. lack of epistasis) occurs between two loci that were both heterozygous. In mosquitoes, Harstone and colleagues [249] showed multiplicative interactions between *kdr* and P450 detoxification in *Culex pipiens quinquefasciatus* whether the resistance alleles were homozygous or heterozyges. For example, resistance ratio 50 (i.e. LC50 resistant strain/LC50 susceptible strain) to permethrin in the double homozygote mosquitoes (RR50 of 30,000) was much higher than that expected (RR50 of 1,400) by simple additive effect of the two loci. Overall, interactions between independent resistant genes are complex. It is therefore important to better understand the interactions between resistant loci as well as to address how the fitness costs/benefits of the mechanisms can manipulate the observed interactions.

Fitness cost

Populations of insects that have never been exposed to insecticides are usually fully susceptible, and resistance genes within those populations are very rare. This usually occurs through

a “fitness cost”, which means that insects sharing the resistance allele lack some other attribute or “quality” such that it gives an advantage to the susceptible insects in an insecticide free environment [250]. For example, resistant insects may have lower mating success, be more susceptible to natural enemies [251], or more prone to mortality during over-wintering [252]. Increased production of metabolic enzymes generally shows lower associated fitness cost than those associated with alterations in the structural genes most probably because the primary function of the enzyme is not disrupted [253]. There is good laboratory and field evidence to suggest that the deficit of insecticide selection pressure, in most cases, selects for susceptible genotypes. For example, the absence of homozygote’s resistant genotypes in *An. gambiae* populations in West Africa is most probably due to the strong genetic cost associated with the carbamate-resistance allele a*ce.1*^{R} (G119S) [254]. In addition, once resistance in the field has been selected it often rapidly reverts once the insecticide treatment regime is changed. A good example of this occurred in *An. arabiensis* in Sudan, where malathion specific insecticide resistance was selected in the early 1980s through antimalarial house spraying. The development of resistance prompted a switch of insecticide treatment to fenitrothion and the malathion resistance rapidly reverted in the following years. However, reversion rates are variable and may be very slow, particularly when an insecticide has been used for many years. For example, DDT was used extensively for malaria control over a 20 year period up to the 1960s in Sri Lanka to control *An. culicifacies s.l.* and *An. subpictus*. DDT was replaced by malathion in Sri Lanka in the early 1970s when a total and effective ban on DDT use was implemented. Subsequent regular monitoring has shown that DDT resistance has reverted very slowly towards susceptibility; around 80% of the adult mosquito population was resistant in the 1970s compared to about 50% in the 1990s. The same is true with the R*dl* gene that was maintained in field mosquito populations despite the abandon of cyclodiene for mosquito control for more than 30 years [33]. Rate of reversion is an important parameter to consider before implementing any resistance management strategy in the field.

8.1.3. Operational factors

In practice, only operational factors such as the insecticide(s) used, the area of coverage (for example for IRS or LLIN), and the timing, rate, and method of application can be manipulated directly to reduce the selection pressure for resistance. Operational factors influence selection by determining the overall fraction of a population exposed (larvae/adults) to a selecting agent and the degree of contact and pick-up of toxicant by exposed pests at what has been termed the "interface between insects and insecticides” [242]. At both stages, operational and intrinsic factors interact in complex ways to establish the net effect of a control treatment on both genetic composition and total population size. Management of resistance therefore entails resolving these interactions to anticipate with some confidence both the suppressive and selective effects of potential control strategies.

Frequency of application, dosage and persistence of effect

How often an insecticide is used is one of the most important factors that influence resistance development [240]. With each use, an advantage is given to the resistant insects within a population. The rate of increase of resistance on any population will generally be faster in the

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presence of a lower fitness cost and high reproductive and short life cycles producing several generations per season. The length of time that an insecticide remains effective, also called its persistence, is dependent upon the physical chemistry of the insecticide, the type of formulation, the application rate and the substrate. Products which provide a persistent effect provide continual selection pressure in a similar manner to multiple treatments. For example, a space spray will persist for a very short time and will select only against a single generation of mosquitoes. In contrast, a residual wall application (IRS) or Insecticide Treated nets treatment (especially Long Lasting Nets) will persist for months or years providing a selection pressure against many generations of the same insect. For example, repeated application of DDT for indoor residual spraying has contributed to increase the number of DDT-resistant malaria vector species in various geographical settings [255]. Several studies showed however that the use of insecticides in agriculture play a key role in the selection of resistance in mosquitoes [256, 257]. Indeed, most insecticides used in agriculture are of the same chemical classes and have the same targets and modes of action as those used in public health programme. In practice, VC programmes cannot influence the choice of the pesticide used for crop protection and the only thing that can be done is to appropriately select the most judicious insecticide for mosquito control. However, there is more published evidence that public health insecticides can contribute to select for pyrethroid resistance alleles (see section 7 for details). It is obvious that we can expect enhanced selection pressure on resistance genes through the scaling up of LLIN and/or IRS for malaria elimination.

Choice of the insecticide

The speed at which an insecticide effectively kills an insect can also influence the evolution of resistance. All current insecticides approved for ITNs or IRS kill extremely rapidly after contact. While fast-acting conventional insecticides can produce even more effective initial control, they impose enormous selection for resistance by killing young female adults. The consequence is that spectacular initial mosquito control can last as little as a few years, thus providing very poor medium- to long-term disease control [258]. Some authors recently suggest that Late Acting insecticides (e.g. entomofungus) may be a more tactical strategy to manage resistance if female mosquitoes are killed after 2 or more gonotrophic cycles [259]. Indeed, the less the insecticide impact on mosquito fitness, the less the strength of selection, especially if the resistance allele is associated with a strong genetic cost. In theory, it would be possible to create an insecticide that would provide effective malaria control yet never be undermined by the evolution of resistant mosquitoes. However, further studies are required as "proof of principle" i.e. to demonstrate that this strategy can be effective for vector management and malaria prevention in a real setting.

8.2. Resistance management — Strategies and tactics

Historically, the practice of using an insecticide until resistance becomes a limiting factor has rapidly eroded the number of suitable/available insecticides for vector control. Rotations, mosaics, and mixtures have all been proposed as resistance management tools [260, 261] but there are very few "success stories" in public health. Numerous mathematical models have been produced to estimate how these tools could be optimally used [262-264] but these models

have rarely been tested under field conditions due to the practical difficulties in estimating changes in resistance gene frequencies (especially for metabolic resistance) in large samples of insects [220]. With the advent of different molecular techniques for resistance-gene frequency estimation, field trials of resistance management strategies have now become more feasible.

8.2.1. Approaches to resistance management

Ideally insecticide resistance management should be undertaken using insecticide based approaches in conjunction with other non-insecticidal vector control methods, i.e. as part of Integrated Vector Management (IVM[4]) [265]. The insecticides used have to be safe to humans and comply with WHO specifications. In practice, most of IVM programmes work well in experimental trials but become challenging when programmes are scaling up into long-term (operational) control. Operationally, the simplest form of resistance management is likely to be "insecticide" based, and this could take several forms.

Rotations

Rotational strategies are based on the rotation over time of two or preferably more insecticide classes with different modes of action. This approach assumes that if resistance to each insecticide is rare, then multiple resistances will be extremely rare [266]. Rotation allows any resistance developed to the first insecticide to decline over time when the second insecticide class is introduced. As for other strategy, rotations are particularly effective if the resistance gene has an associated fitness cost. The timeframe for rotation needs to be sufficiently short to prevent significant levels of resistance to develop to any one rotation partner. Rotations have been successful in many applications in agriculture and are considered to be effective in slowing the evolution of resistance (see [240] for details). The most striking example of "success story" using this strategy was within the framework of the Onchocerciasis Control Programme (OCP) carried in West Africa 40 years ago. Indeed, weekly application of unrelated larvicides in rivers was successful to kill the larvae of the blackfly vector and mitigate the spread of temephos resistance over the 17 years of its implementation [267]. However, the rotation was introduced at early stage of the OCP, as soon as the operators faced temephos resistance problems in pilot localities. As for all IRM strategies, the status of resistance of the insecticide used in the rotation must be known when implementing rotations and the chemicals used should not present any (known) cross-resistance. For LLIN, it is difficult to implement this method knowing that only pyrethroids are recommended so far by WHO for the impregnation [268]. For IRS, the pragmatic approach would be to rotate insecticides annually. Indeed, changing insecticides more than once a year (which could be the case in areas where two spray rounds are conducted each year) is not recommended, because of procurement and other financial and logistical challenges (see [4]). Despite higher cost of implementing rotation than single spray (as available alternatives to pyrethroids —the carbamates organophosphates, insect growth regulators, pyroles —are currently more expensive), this is probably the price to pay to preserve the arsenal of cost-effective insecticides for malaria vector control.

4*Integrated Vector Management* can be defined as "a rational decision making process for the optimal use of resources for vector control". IRM is therefore an integral part of IVM, as only through the active management of insecticide resistance can the available resources be optimally and sustainably used.

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Mosaics

Spatially separated applications of different compounds against the same insect constitute a "mosaic" approach to resistance management [240]. Fine scale mosaics can be achieved in malaria vector control programmes, for example, by using two insecticides in different dwellings within the same village. The aim of this strategy is to preserve susceptibility by spatial restriction of insecticides [4]. If such a fine scale mosaic is to be used, careful records of which insecticide was used in each house are essential. Larger scale mosaics have been shown to be effective for the management of pyrethroid resistance in *An. albimanus* in Mexico [22]. Indeed, pyrethroid resistance rose more rapidly in the areas under pyrethroid treatment alone than in the mosaic areas using OP, Pyrethroid and carbamate [240]. Whilst there are some practical difficulties implementing a mosaic in a vector control programme (eg spray with different insecticides, dosages, apparatus, etc), it may offer the advantages of a mixture strategy with lower insecticide inputs and hence cost. The scale at which a mosaic needs to be applied has not been clearly established. In South Africa for example, different insecticides have been used in different types of houses within the same community and this is considered by some to be a mosaic-like strategy [240]. Similarly, mosquito bed nets from panels treated with different insecticides achieve a similar mosaic effect to treating houses with different compounds but on a much finer scale. Industry has recently developed mosaic LLINs containing a pyrethroid insecticide and a synergist (Piperonyl butoxide or PBO an oxidase inhibitor) on the roof to increase efficacy against pyrethroid-resistant malaria vectors. Small scale field trial [235, 269] and mathematical exercises [270] suggested that mosaic LLIN may provide better insecticidal effect against resistant mosquitoes and enhanced community-level protection against malaria compared to "classical" LLIN in area of pyrethroid resistance. Clearly further operational research is required to establish the applicability and effectiveness of mosaics approaches for malaria control.

Mixtures

A mixture is defined by the simultaneous use of two or more insecticides of unrelated mode of action. If two insecticides A and B, with independent resistance mechanisms, are applied together in a mixture, and if resistance to A and resistance to B are both rare, then we expect doubly resistant insects to be extremely rare, and almost all insects resistant to A will be killed by B, and vice versa [266]. This system of "redundant killing" means that resistance to the two insecticides will evolve much more slowly than if either had been used on its own [271]. This approach may be not successful if resistance to one of the components used is already present at a detectable level and/or if linkage disequilibrium is present in the targeted population [4]. Unlike rotations, the effectiveness of mixtures is not directly related to the degree of fitness cost. Rather the mixture aims to overpower resistance instead of preserving susceptibility. However, for mixtures to work well in practice both insecticides need to be used at their full application rate in order that the efficacy and persistence of the two insecticides would be broadly similar (same decay rate). Further, theoretical models suggest that mixtures might delay resistance longer than rotations or broad mosaics [271, 272]. However, mixtures of products were rarely adopted in malaria vector control programmes on grounds of cost, logistics, and safety issue and because of the limited number of recommended compounds available for both

IRS and LLIN. It is not yet clear however how much the addition of a second active ingredient will add to the total cost of manufacturing since the cost of additional insecticide can greatly vary according to the strategy, ie. cost for LLINs would be much lower than that for IRS. For LLIN, previous laboratory and field trials showed interesting prospects for reducing mosquito survival and biting rates with the use of insecticide mixtures applied on mosquito nets against *kdr*-resistant *An. gambiae* in Africa [273, 274]. Other chemicals, such as insect growth regulators (IGR), represent also promising alternative to be included in mixture formulations as they may impact on mosquito longevity, fertility and fecundity [275, 276]. With the development of next-generation of LLIN, combined use of non-pyrethroids and pyrethroids on bed nets is technically achievable and has the potential to provide better control of malaria and prevent further development of pyrethroid-resistance in malaria vectors. Risk assessment and acceptability of such new tools should be however carefully investigated before any trial being implemented at operational level.

Combinations

In this context, combinations expose the vector population to two vector control tools, such that a mosquito that survives contact with one (e.g. LLIN) is exposed to the other one (e.g. IRS), or vice versa. In practice, exposure to two insecticides is not guaranteed but there is some evidence to indicate that this is likely [277]. The effectiveness of combinations in IRM does not depend on the ability to reduce the level of resistance, but on the ability to kill the vector despite the existence of resistance, through the use of another insecticide or intervention, which compensates for resistance [231]. As for other strategy, the combination should not contain insecticides with same mode of action (e.g. avoid pyrethroids for both IRS and LLINs), as this would increase selection pressure rather than reducing it. As combinations require doubling of interventions, cost would be significantly higher than rotations and mosaics. This might nevertheless be warranted in some circumstances, for example where malaria transmission is very high and/or where targeted IRS can help overcome identified resistance to pyrethroids in areas with high LLIN coverage. In practice, combinations would be more easily implemented in countries having sufficient human and financial resources allocated to public health programmes. So far, a small number of observational studies [278-280] and mathematical modeling exercises [263, 264] suggest that VC combination has an added benefit for reduction of the risk of infection because the people not protected by one of the interventions are protected by the other. A recent cluster randomized controlled trial carried out in Benin showed however that neither clinical malaria in children younger than 6 years nor transmission intensity differ between LLIN and carbamate-IRS or Carbamate Treated Plastic Sheeting and the reference group (LLIN alone) and the insecticide combinations did not slow down the evolution of the *kdr* allele in *An gambiae s.s.* compared with LLIN [231]. It was concluded from this study that IRS should be timely implemented (i.e. using appropriate insecticide, dosage and time interval) to ensure optimum efficacy of the IRS intervention over LLIN. Clearly, cost-effectiveness of combined vector control interventions need to be carefully considered to ensure that increased efforts and cost dedicated to combinations effectively contribute to better control and management of pyrethroid-resistant malaria vectors.

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9. Conclusions

Insecticide resistance develops in an insect population when individuals carrying genes that allow them to survive exposure to the insecticide pass these genes on. Thus, any activities that control the individuals with the resistance trait will delay the spread of the resistance genes in the population. IRM should then be seen in the context of IVM and should therefore also include activities such as habitat management, community education, and/or larval source management (e.g. biological control). In order to successfully develop and implement any resistance management strategies based on rotations, mosaics, mixtures or combinations, knowledge of the mode of action, chemical properties, and residual life of the available insecticide products is essential. Although insecticides with novel modes of action have recently been introduced in public health (neonicotinoids, pyroles, oxadiazin, etc) few of them appear to have the optimum biological and/or physical properties required for residual wall spray and/or mosquito net. Unfortunately, the exorbitant costs associated with developing and registering new insecticides (see [281] for details) mean that products appear in the more profitable agricultural markets before consideration is given to their public health potential. We have then no other option than to make an appropriate and judicious use of the current insecticides if we want to avoid any disillusion with pyrethroids as we faced before with DDT or dieldrin. The philosopher George Santayana said *"those who cannot remember the past are condemned to repeat it."* Hope it's not too late for malaria vector control.

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