Review

Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?

Hilary Ranson[1], Raphael N'Guessan[2,5], Jonathan Lines[3], Nicolas Moiroux[4,5], Zinga Nkuni[3] and Vincent Corbel[4,5]

[1] Vector Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK, L3 5QA
[2] London School of Hygiene and Tropical Medicine, Keppel Street, London, UK, WC1E 7HT, UK
[3] Global Malaria Programme (GMP), World Health Organization, 1211 Geneva 27, Switzerland
[4] Institut de Recherche pour le Développement (IRD), RU016 « Caractérisation et Contrôle des Populations de Vecteurs », 01 BP 4414, Cotonou, Benin
[5] Centre de Recherche Entomologique de Cotonou (CREC), Laboratoire National, Ministère de la Santé, Cotonou 06 BP 2604, Benin

The use of pyrethroid insecticides in malaria vector control has increased dramatically in the past decade through the scale up of insecticide treated net distribution programmes and indoor residual spraying campaigns. Inevitably, the major malaria vectors have developed resistance to these insecticides and the resistance alleles are spreading at an exceptionally rapid rate throughout Africa. Although substantial progress has been made on understanding the causes of pyrethroid resistance, remarkably few studies have focused on the epidemiological impact of resistance on current malaria control activities. As we move into the malaria eradication era, it is vital that the implications of insecticide resistance are understood and strategies to mitigate these effects are implemented.

The importance of pyrethroid insecticides for malaria control

Insecticide treated nets (ITNs) and indoor residual spraying (IRS) are the cornerstone of malaria control programmes, [1] and high coverage with either of these interventions can result in a dramatic reduction in malaria associated morbidity and mortality [2,3]. The success of these tools has contributed towards the optimism that elimination of malaria as a public health problem in the African continent is a feasible objective [4].

Unfortunately the public health pesticide products market has suffered from massive under investment with the result that there have been no new classes of active ingredients available for wide scale public health applications for more than 30 years. Hence the emergence of resistance to the majority of existing insecticides is in danger of undermining the contribution of vector control efforts.

Malaria vector control is currently very dependant on a single class of insecticides, the pyrethroids. These insecticides are the only class approved for use on insecticide treated nettings [5] and are being increasingly deployed in IRS programmes in Africa. Pyrethroids are also widely used in the control of agricultural pests worldwide [6]. There has been a dramatic increase in reports of pyrethroid resistance in malaria vectors over the past decade [7], but few studies have addressed the impact this is having on malaria control; controversy still remains about the epidemiological significance of current levels of resistance in Sub-Saharan Africa. Current understanding of the mechanisms responsible for pyrethroid resistance in malaria vectors are outlined below and alternative methods of resistance monitoring critically appraised. The current distribution of pyrethroid resistance in malaria vectors in Africa and the available evidence on the entomological and epidemiological impact of this resistance are reviewed.

Resistance

Typically two major mechanisms are assumed to be responsible for insecticide resistance (Box 1): changes in the insecticide target site that reduce its binding and increases in the rate of insecticide metabolism that lower the amount of insecticide reaching the target site. Of these, 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 some malaria control programmes [8,9]. 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. Although these two mechanisms clearly play a major role in conferring pyrethroid resistance, consideration of other physiological or behavioural changes in the mosquito population that might impact on the efficacy of pyrethroid insecticides is also important. Two potential mechanisms that have been long recognised, but whose importance in malaria vectors has been largely overlooked are cuticular and behavioural resistance (Box 1).

Target site resistance

The pyrethroid insecticides (and the organochlorine insecticide DDT) target the voltage-gated sodium channel on the insects' neurons [10]. Insecticide binding delays the closing of the sodium channel prolonging the action potential and

Corresponding author: Corbel, V. (Vincent.corbel@ird.fr).

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Box 1. Definitions of resistance

Insecticide resistance (WHO): 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.

Operational (field) resistance (Insecticide Resistance Action Committee, IRAC): defined 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.

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.

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.

Phenotypic resistance: expression of a resistance gene on an insect life history trait (e.g. survival rate, knock down, etc.).

Target site resistance: changes in the sensitivity of insecticide target due to non-silent point mutations.

Metabolic resistance: overexpression of enzymes capable of detoxifying or sequestering insecticides and/or amino acid substitutions within these enzymes which alter the affinity of the enzyme for the Insecticide.

Behavioural resistance: describes any modification in insect behaviour that helps to avoid the contact and/or lethal effects of the insecticides.

Cuticular resistance: modifications in the insect cuticle and/or digestive tract linings that prevent or slow down the absorption or penetration of insecticides.

causing repetitive neuron firing, paralysis and eventual death of the insect. Alterations in the target site that cause resistance to insecticides are often referred to as knock-down resistance (kdr) in reference to the ability of insects with these alleles to withstand prolonged exposure to insecticides without being 'knocked-down.' Several mutations in the sodium channel have been associated with resistance to pyrethroids in a variety of insects [10]. One of the most common amino acid replacements, and so far the only residue associated with pyrethroid resistance in malaria vectors, is a substitution of the leucine residue found at codon 1014 with either phenylalanine (1014F) or serine (1014S). Interestingly, residue 1014 does not appear to interact directly with the insecticide but is predicted to alter channel activation kinetics [11].

The relationship between the kdr genotype and the resistance phenotype in malaria vectors has recently been extensively reviewed and will not be discussed in detail here [12]. In summary, it is clear that kdr is associated with resistance to pyrethroids and DDT, but it is not evident that the presence of this resistance allele alone is sufficient to result in control failure.

Metabolic resistance

Metabolic resistance occurs when elevated activities of one or more enzymes results in a sufficient proportion of the insecticide being sequestered or detoxified before it reaches the target site to impair the toxicity of the insecticide. The cytochrome P450 s are the primary enzyme family responsible for pyrethroid metabolism in insects [13]. There are 111 P450 enzymes in Anopheles gambiae [14] and, as in other insects, only a small number of these enzymes are capable of detoxifying insecticides. Microarray-based approaches have identified three 'candidate' P450 genes that were found to be repeatedly over expressed in pyrethroid resistant populations of An. gambiae: cyp6m2, cyp6p3 and cyp6z2 [15–17]. All of these genes encode for enzymes that are able to bind to pyrethroid insecticides, but only CYP6P3 and CYP6M2 can metabolise the insecticide [17,18]. Interestingly recent studies in Anopheles funestus have identified the putative ortholog of An. gambiae cyp6p3, cyp6p9, as being the prime candidate for conferring pyrethroid resistance in this species [19]. Functional genomics approaches are needed to conclusively demonstrate their role in resistance.

Other enzyme families might also play a secondary role in pyrethroid resistance by, for example, protecting from pyrethroid exposure induced oxidative stress [20], detoxifying secondary products of P450 based metabolism [21], or binding insecticides to lower the total *in vivo* concentration of insecticide [22]. The contribution that these enzymes make towards the pyrethroid resistance phenotype in malaria vectors is yet to be elucidated.

The absence of molecular markers for metabolic resistance makes it difficult to directly assess the impact of this resistance on vector control interventions. The situation is complicated by the co-occurrence of kdr and metabolic resistance in many vector populations. However, the population of malaria vectors that has proved most intransigent to control with pyrethroid insecticides is An. funestus from Southern Mozambique [23]. Target site resistance is not found in this population, and instead, resistance appears to be caused by the massive overexpression of a small number of P450 enzymes [19]. Extensive efforts are underway to identify the causal mutations associated with metabolic resistance to pyrethroids in the major malaria vectors [24].

Cuticular resistance

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. Hence 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 insecticide *in vivo*. Intriguingly microarray experiments have identified two genes, cplcg3 and cplcg4, encoding cuticular proteins that are upregulated in pyrethroid resistant strains of Anopheles mosquitoes from three populations and two species [25,26]. Clearly much more work is required in order to identify the significance of cuticular resistance.

Behavioural resistance

There have been several anecdotal reports of mosquitoes changing their behaviour as a result of intensive indoor use of insecticides, but there is currently insufficient data to assess whether these behavioural avoidance traits are genetic or adaptive responses [27]. Genetic changes in the malaria vector population that shifted feeding or

Box 2. Methods for detecting 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. The World Health Organisation (WHO) has defined diagnostic doses for most insecticides used in malaria control and produces susceptibility test kits consisting of exposure chambers and insecticide treated filter papers. Guidelines for test procedures and interpretation of results are available from the WHO (see http://www.who.int/whopes/resistance/en/). Although simple to perform, these diagnostic dose assays provide limited information and several alternative methods for detecting resistance are available (Table I). 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 methods in Table I are suitable for detecting behavioural resistance.

Table I. Alternative methods to detect insecticide resistance in mosquitoes

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)
Dose response bioassays	Provides data on level of resistance in population, regardless of mechanism	Require large numbers of live 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 material to be kept frozen. 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.

resting behaviour to minimise contact with insecticides in the indoor environment could have a very dramatic impact on the efficacy of current malaria vector control interventions, potentially exceeding the impact of physiological resistance. There is a clear need for robust controlled studies to quantify the extent of this behavioural change and to assess whether scale-up of ITNs and/or IRS could increase the importance of outdoor transmission of malaria and necessitate new tools to target malaria vectors that are feeding, and/or resting, outdoors.

Cross-resistance pattern

Understanding patterns of cross resistance caused by alternative mechanisms is vital to the implementation of effective resistance management strategies. It is generally assumed that resistance renders the selecting insecticide, and all others with a similar mode of action, ineffective. For example, the high frequency of kdr mutations in malaria vectors is often attributed to extensive past use of DDT to control agricultural pests in Africa [28]. This assumption might hold true for target site resistance, but this is not necessarily the case for metabolic resistance mechanisms. CYP6P3 and CYP6M2 are efficient at detoxifying pyrethroids but current evidence suggests they do not metabolise DDT (Paine, personal communication). Furthermore, some P450 enzymes show specificity for either type I pyrethroids (those lacking a cyano group, such as permethrin) or type II pyrethroids (containing an alpha-cyano group, e.g. deltamethrin) [29]. In the alternative scenario, metabolic resistance might confer resistance to more than one class of insecticides. For example, a pyrethroid resistant strain of An. funestus from Mozambique shows cross resistance to carbamate insecticides, and synergist data suggests that cytochrome P450 s are responsible for both phenotypes [30]. Whether the same enzyme is capable of metabolising both insecticide classes is currently unknown but this information is clearly of key importance if rotations or mixtures with non pyrethroids are employed as part of a resistance management programme.

Detecting and reporting 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 allowed to build up unchecked, resistance can eventually become 'fixed' in the population as initial detrimental effects on the insect's fitness are overcome by compensatory mutations. Once resistance reaches very high levels, strategies to restore susceptibility are unlikely to be effective. Some alternative approaches for detecting insecticide resistance are described in Box 2.

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 [31]. It is clear that resistance is a dynamic trait, and wide fluctuations in resistance levels throughout the malaria transmission season have been reported [32]. Resistance can also be very focal, particularly when vector composition differs between sites [33], hence a minimum number of sampling sites should be established, taking into account patterns of vector distribution and insecticide usage.

The World Health Organisation African Network for Vector Resistance (ANVR) 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 ANVR members, but this database was not readily accessible by outside users. The recent

establishment of IRBase [34] as an online centralised 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 is more readily available to all interested parties.

Current status of pyrethroid resistance in African malaria vectors

The maps in Figure 1 summarise the current published literature on the distribution of pyrethroid resistance and their underlying mechanisms in malaria vectors in Africa. Bioassay data was available for 23 out of 49 African countries investigated, but the distribution of data is not uniform (Figure 1a). Pyrethroid resistance in An. gambiae was first reported in Cote d'Ivoire in 1993 and is now widespread throughout the western [35,36] and central regions [7,37] of Africa. In Eastern and Austral Africa, An. gambiae s.s. and An. arabiensis populations are mostly susceptible in Tanzania [38], Mozambique [39] and Madagascar [40], but pyrethroid resistance has been reported in Uganda [41,42], the Gwave region of Zimbabwe [43], Sudan [44,45] and Ethiopia [46,47].

There is a paucity of published data on insecticide resistance in An. funestus, but resistant populations have been reported in South Africa [23], Mozambique [30]

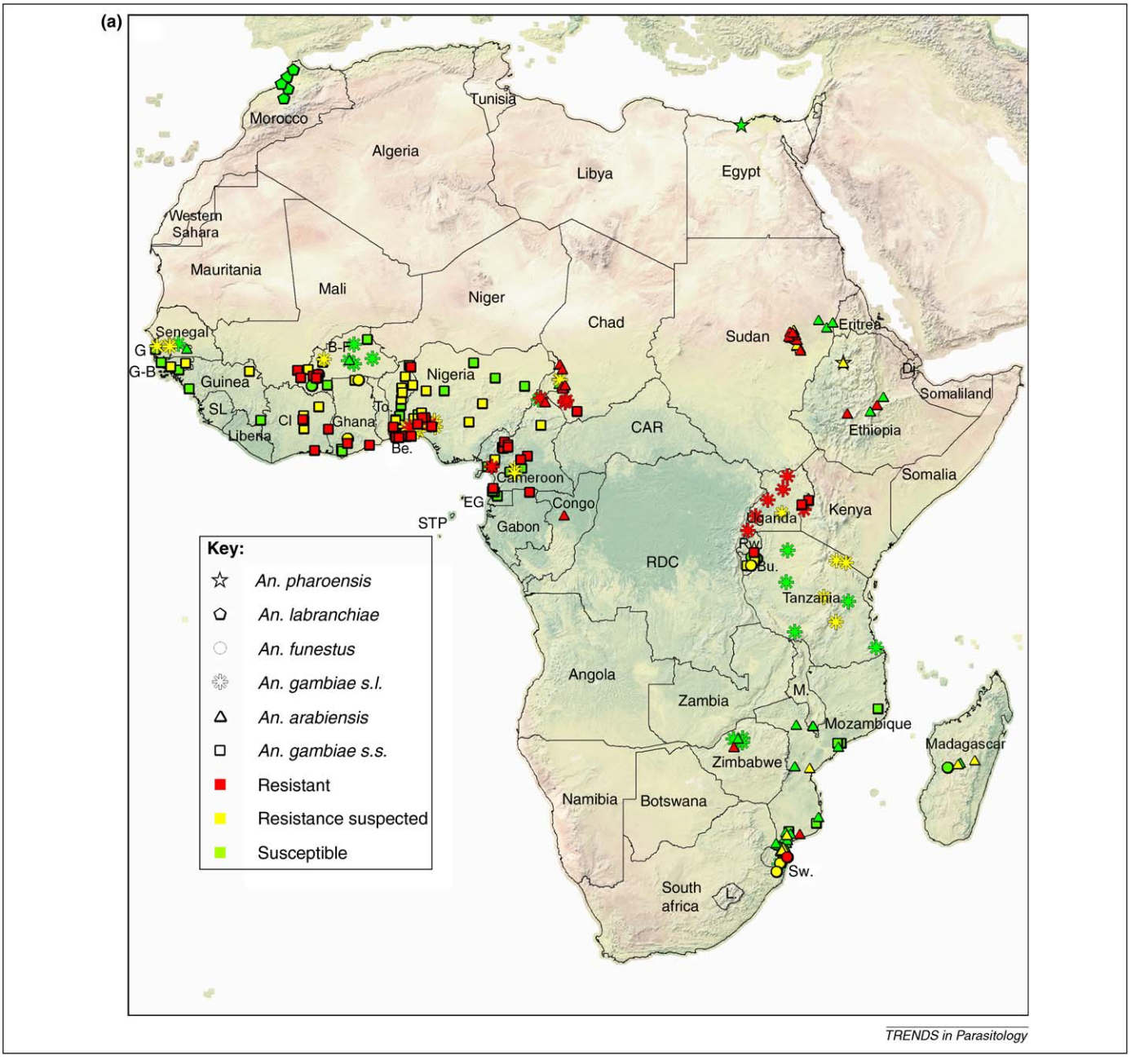

Figure 1. Map of Africa showing the distribution of pyrethroid resistance **(a)** and their underlying mechanisms **(b)** in malaria vectors. Maps were built using data collected from 2000 to 2010. Sites were located using publications (see references and references cited therein) and the Geonames database of the National Geospatial-Intelligence Agency, and spatialized using the ESRI ArcGis Software. Background and boundary data are freely available at www.naturalearthdata.com. Resistance was defined according to standard WHO definitions, as described by the WHO [78]. Metabolic resistance data were incorporated when the field populations showed significantly higher enzyme activity and/or gene expression levels than that of the reference susceptible strain (using biochemical assays and/or detox chip microarrays). Target site resistance is recorded wherever the 1014F and/or 1014S kdr alleles have been reported but the maps do not provide any indication of the frequency of these resistant alleles in each site.

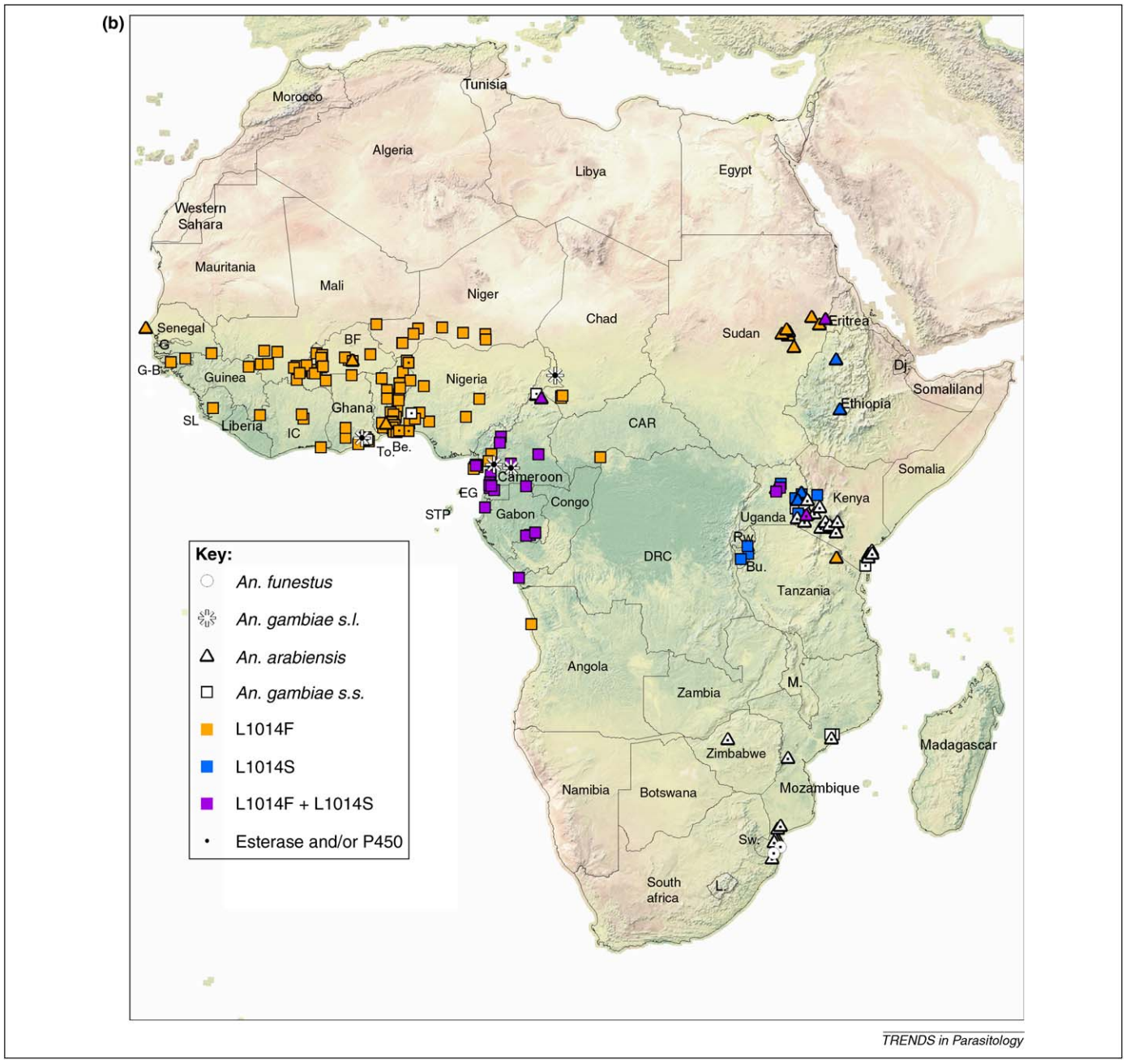

Figure 1. (*Continued*).

Ghana [48] and Burundi [49]. Full susceptibility to pyrethroids has been reported in An. labranchiae in Morocco [50] and in An. pharoensis in Egypt [51] and Ethiopia [52], and there have been no reports of resistance in other secondary vectors.

The current understanding of the distribution of kdr alleles is shown in Figure 1b. The 1014F mutation was first detected in An. gambiae from Cote d'Ivoire [53]. Subsequently 1014S was detected in An. gambiae from Kenya [54]. This somewhat misleadingly led to these two mutations being referred to as kdrWest and kdrEast. It is now clear, however, that these alleles are not restricted to either side of the continent. In An gambiae s.s. the 1014F mutation has been detected as far East as Uganda [46] and the 1014S mutation has been found in Angola [7] and several countries in central Africa [9,55] (Figure 1b). There have also been several reports of 1014F/1014S hybrids in An gambiae s.s. [42,56–59].

Both 1014F and 1014S alleles have been detected in the sister taxa, An. arabiensis [45,60]. To date kdr does not appear to have arisen in An. funestus since both genetic mapping and direct sequencing of the sodium channel in pyrethroid resistant populations failed to find any evidence of target site resistance in this species [61]. Outside of Africa, kdr has been found in several malaria vectors including An. stephensi and An. culicifacies [62,63].

The absence of simple genetic markers for metabolic resistance means that far less is known about the distribution of the responsible alleles. Biochemical assays, and in some cases microarray studies, have implicated metabolic resistance in An. gambiae s.l. in Kenya [64], Camer-

oun [65], Benin [15], Nigeria [15], Ghana [16], Mozambique [39], South Africa [66] and Zimbabwe [43]. Overexpression of CYP6P3 and/or CYP6M2 has been found in pyrethroid-resistant An. gambiae populations from Benin, Nigeria and Ghana [15,16], mainly in co-association with the kdr L1014F allele. This co-occurrence of resistance genes might constitute an additional threat to malaria vector control as epistasis between these two types of resistance conferred an extremely high level of pyrethroid resistance in the mosquito Culex quinquefasciatus [67].

Entomological and epidemiological impact of pyrethroid resistance

Few studies have assessed the epidemiological impact of insecticide resistance, and interpreting data from such studies is complicated by the large number of confounding factors. The clearest example of control failure being directly attributed 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 [30]. Within four years, reported malaria cases had increased approximately four-fold, and An. funestus, which had previously been eradicated, had reappeared and was observed emerging alive from pyrethroid sprayed houses. Bioassays showed that this species was resistant to pyrethroids but susceptible to DDT [23]. The decision to revert to IRS with DDT was accompanied by a decline in malaria cases by 91% [68].

On the island of Bioko on the West African coast, an IRS campaign with lambdacyalothrin failed to curtail an increase in the population density of pyrethroid resistant *An. gambiae*; although a modest but significant reduction in transmission index and malaria reported cases was observed [9,69]. High frequencies of the L1014F kdr allele were observed in the local An. gambiae population. Only after pyrethroids were replaced with the carbamate bendiocarb did the mosquito population decline [9]. Nevertheless, in an operational scale programme such as this, the possible contribution of other factors to the failure of pyrethroid IRS to control mosquito population density cannot be overlooked; thus the direct consequence of the high kdr frequency is uncertain.

Another programmatic study was conducted in the highland provinces of Burundi, where a vector control programme combining IRS with pyrethroids and ITNs was initiated in 2002 in one of the most malaria affected island provinces, Karuzi. Here the interventions significantly reduced Anopheles density by 82% and transmission intensity by 90% and occurrence of clinical episodes by 43% in children despite high frequencies of the L1014S kdr allele in the local An. gambiae s.s. [49,70,71].

There have been extensive randomized controlled trials (phase III) in Africa aimed at investigating the efficacy of ITNs for malaria prevention [2], but very few have assessed how pyrethroid resistance might affect the effectiveness of such intervention. In the Korhogo area in the north of Côte d'Ivoire where the 1014F kdr allele frequency in An. gambiae is >80% [53] and malaria is endemic, lambdacyhalothrin-treated nets had a significant impact on the entomological inoculation rate (55% reduction) and on malaria incidence in children under 5 years of age (56% reduction of clinical attacks) compared to a control group having no nets [72]. This was the first clear-cut evidence of ITNs continuing to provide effective personal protection against malaria in an area with a high frequency of kdr in the vector population. However, the absence of a physical barrier in the control group might have overestimated the impact of pyrethroid treated nets against kdr mosquitoes in this study.

In southern Benin, a randomized controlled trial was carried out in a mesoendemic area to assess the impact of long lasting ITN scale-up on malaria morbidity in children under five years of age [73]. In this area, where the kdr frequency is around 50 to 60% in An. gambiae s.s, transmission increased during the rainy seasons but was not followed by a seasonal variation in parasite infection and clinical incidence. A longitudinal survey conducted among sleepers and nonsleepers under ITNs in that area of Benin showed no significant reduction in parasite density or clinical attacks of malaria (Corbel, unpublished data). Clearly further investigation is needed in this area to assess the impact of pyrethroid-resistance on vector control effectiveness.

Other smaller scale studies have assessed the impact of resistance on entomological parameters, using experimental huts, with variable results. An early experimental hut trial of ITNs in Côte d'Ivoire showed no apparent difference in the effectiveness of ITNs between two adjacent sites with resistant and susceptible populations of An. gambiae [75]. By contrast, a comparative study of the efficacy of lambdacyhalothrin used for IRS or net treatment in southern Benin indicated a major loss of efficacy associated with pyrethroid resistance in An. gambiae compared to the north where this species remains largely susceptible to pyrethroids [76].

One of the problems associated with many of these studies is that, owing to the lack of molecular markers for alternative resistance mechanisms, the frequency of kdr alleles is frequently used as a proxy for resistance. This can be misleading if metabolic or other resistance mechanisms are the predominant resistance mechanism. There is an urgent need for properly controlled large-scale trials to assess the impact of pyrethroid resistance on IRS and ITNs, alone or in combination. 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 and monitor for changes in key traits such as location of resting and feeding that can impact on the efficacy of current insecticide based interventions.

Conclusions

Pyrethroid resistance, as measured by conventional bioassays, is clearly widespread in malaria vectors across Africa. Molecular studies tracking the frequency of insecticide resistance alleles have shown dramatic increases in the frequency of these alleles in An. gambiae in recent years, presumably reflecting the increased selection pressure on malaria vectors, caused at least in part by the scale up of ITN coverage and pyrethroid use in IRS.

Enthusiasm for resistance management might be hindered by the paucity of reliable data on the impact of resistance on current interventions, and this must clearly be a priority for further research. Nevertheless, the rapid increase in pyrethroid resistance necessitates an immediate proactive response to resistance management to avoid compromising existing effective interventions. Resistance management is challenging when only a single chemical class is recommended for a particular application, as is the case of bed nets. Even for IRS, with only four insecticide classes currently available (having two different modes of action) and resistance reported to all four of these in some populations of An. gambiae [32], the options for managing resistance and providing sustainable vector control with existing chemicals are limited.

The future is not completely bleak. The Innovative Vector Control Consortium, established in 2005, is a product-development partnership established to stimulate the search for alternative active ingredients or improved formulations of insecticides for vector control, and several promising leads are now being evaluated in laboratory and field trials [77]. However, given the protracted regulatory procedures, it is likely to be many years before these new chemicals are an option for malaria control programmes. Hence it is vital that policy makers and programme implementers recognise the growing threat posed by insecticide resistance and strive to integrate resistance management into all control programmes. In addition, alternative, non-insecticidal methods should be encouraged, wherever feasible, to help reduce the reliance on pyrethroid insecticides.

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