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Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene

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Abstract. Samples of the dengue vector mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) were collected from 13 localities between 1995 and 1998. Two laboratory strains, Bora (French Polynesia) and AEAE, were both susceptible to DDT and permethrin; all other strains, except Larentuka (Indonesia) and Bouaké (Ivory Coast), contained individual fourth-instar larvae resistant to permethrin. Ten strains were subjected to a range of biochemical assays. Many strains had elevated carboxylesterase activity compared to the Bora strain; this was particularly high in the Indonesian strains Salatiga and Semarang, and in the Guyane strain (Cayenne). Monooxygenase levels were increased in the Salatiga and Paea (Polynesia) strains, and reduced in the two Thai strains (Mae Kaza, Mae Kud) and the Larentuka strain. Glutathione S-transferase activity was elevated in the Guyane strain. All other enzyme profiles were similar to the susceptible strain. The presence of both DDT and pyrethroid resistance in the Semarang, Belem (Brazil) and Long Hoa (Vietnam) strains suggested the presence of a knock-down resistant (*kdr*)-type resistance mechanism. Part of the S6 hydrophobic segment of domain II of the voltage-gated sodium channel gene was obtained by RT-PCR and sequenced from several insects from all 13 field strains. Four novel mutations were identified. Three strains contained identical amino acid substitutions at two positions, two strains shared a different substitution, and one strain was homozygous for a fourth alteration. The leucine to phenylalanine substitution that confers nerve insensitivity to pyrethroids in a range of other resistant insects was absent. Direct neurophysiological assays on individual larvae from three strains with these mutations demonstrated reduced nerve sensitivity to permethrin or lambda cyhalothrin inhibition compared to the susceptible strains.

Key words. *Aedes* aegypti, carboxylesterase, cross-resistance, DDT resistance, *kdr* mutation, knockdown resistance, pyrethroid resistance, voltage-gated sodium channel, Brazil, French Guiana, French Polynesia, Indonesia, Ivory Coast, Martinique, Thailand, Vietnam.

Introduction

There are two major mechanisms of pyrethroid resistance in insects: increases in the rate of metabolic detoxification of the insecticide, or changes in target site sensitivity. Metabolic detoxification is often associated with changes in

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monooxygenase activity, producing pyrethroid-specific resistance (Berge *et al.*, 1998), but non-specific esterases and elevated glutathione-S-transferases have also been reported to confer pyrethroid resistance (Vulule *et al.*, 1999; Vontas *et al.*, 2001). The common target site for pyrethroids and DDT is the voltage-gated sodium channel, and *kdr*-like mutations in this gene have been linked to changes in sensitivity of this target in a range of insects, including the mosquito *Anopheles gambiae* (Ranson *et al.*, 2000; Martinez-Torres *et al.*, 1998). Cross-resistance between insecticide classes occurs with the target-site mechanism.

There are numerous reports of pyrethroid resistance in the major dengue vector *Aedes aegypti* (Ziv *et al.*, 1969; Malcolm & Wood, 1982; Hemingway *et al.*, 1989; Mebrahtu *et al.*, 1997). Many of the early reports were of cross-resistance to pyrethroids from field selection with DDT, implicating a *kdr* mechanism (Hemingway *et al.*, 1989), although some early reports of larval DDT resistance in *Ae. aegypti* were linked to increased levels of metabolism (McDonald & Wood, 1979). Target-site resistance has been inferred in *Aedes* through cross-resistance and neurophysiological studies (Hemingway *et al.*, 1989), but the molecular basis of the target-site changes are unknown. In several other insect species, the most common *kdr* mutation associated with target-site resistance is a leucine to phenylalanine (Leu → Phe) substitution in the S6 hydrophobic segment of domain II in the sodium channel gene (Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1998). However, other changes at the same site, such as the leucine to serine substitution in East African *An. gambiae*, have been demonstrated (Ranson *et al.*, 2000). An additional methionine to threonine replacement occurs between the S4 and S5 segments of domain II in houseflies and hornflies. In houseflies the combination of this mutation and the Leu → Phe alteration confers higher levels of pyrethroid resistance, referred to as *super kdr* (Jamroz *et al.*, 1998). In highly resistant German cockroaches this methionine to threonine replacement is absent, but up to four other novel mutations in addition to the Leu → Phe mutation have been reported (Liu *et al.*, 2000). Two of these were found in the linker connecting domains I and II, whereas the other two mutations were located at the N- and C-termini. Sequencing of the sodium channel gene from temperature-sensitive *Drosophila* mutants, which also have insensitivity to pyrethroid binding on the nerve membranes, has identified mutations in homologous position to both *kdr* and *super kdr* in domains I and III, and at a novel position in the loop between the S5 and S6 segments of domain III (ffrench-Constant *et al.*, 1998). None of the *Drosophila* temperature-sensitive mutants mapped to domain II. Field-caught pyrethroid-resistant *Heliothis virescens* have a *kdr*-like leucine replacement located in the equivalent of the S6 segment of domain I (Park & Taylor, 1997). Hence, functional sodium channels with pyrethroid insensitivity may result from one (or more) of several potential amino acid substitutions. This contrasts with cyclodiene resistance, where a single alanine to serine substitution in the GABA receptor of the chloride channel is invariably seen (ffrench-Constant *et al.*, 1998).

Despite its generally limited foraging range, *Ae. aegypti* is more prone to dispersal than most other mosquitoes, as it may be inadvertently transported as dried eggs in a range of containers. Hence, it is a species in which a single resistance mechanism could spread rapidly, as has been documented in *Culex quinquefasciatus* Say (Raymond *et al.*, 1991). This study was undertaken to determine the biochemical and molecular basis of pyrethroid resistance in *Ae. aegypti* from a wide variety of geographical locations.

Materials and methods

Thirteen strains of *Ae. aegypti* were collected between 1995 and 1998 from the field as follows: Belem (1°26′ S 48°30′ W) Brazil; Bouaké (7°41′ N 5°02′ W) Ivory Coast; Cayenne (4°55′ N 52°18′ W) French Guyana; Baniona (8°20′ N 123°15′ E), Larentuka (8°20′ N 123° E) and Semarang (6°58′ S 110°25′ E), Indonesia; Marlebel (14°40′ N 61° W) Martinique; Mae Kaza and Mae Kud (13°45′ N 100°5′ E) Thailand; Long Hoa (10°15′ N 107°10′ E) Vietnam; recently colonized strain from Salatiga (7°18′ S 110°28′ E), Indonesia, and from Bora (16°30′ S 151°45′ W) and Paea (17°32′ S 149°34′ W), French Polynesia. For comparison we used the long-standing AEAE-susceptible reference strain. For each new strain, several hundred larvae and pupae were collected from outdoor 200 L metallic drums and indoor concrete basins for domestic showers, and also from coconut shells in Paea. Emerged adults were mated and their F1 eggs were taken to IRD Montpellier, France, for laboratory investigations. Fifty insects from each strain were used for each of the biochemical assays described below, from the F1 generation for most strains (except Mae Kaza and Mae Kud). Strains were bred in the laboratory for one or two generations. Susceptibility to the insecticides DDT, permethrin (25% *cis* 75% *trans*, Agrevo, Frankfurt, Germany) and lambda-cyhalothrin was determined in F2 or F3 adults following standard WHO susceptibility testing protocols (WHO, 1970; WHO, 1980) or by exposure of fourth-instar larvae to serial dilutions of insecticide in aqueous media (WHO, 1981). Overall, 5000 insects were used for these bioassays.

Glutathione S-transferase (GST) activity

A 1 mL reaction mixture in phosphate buffer (50 mM, pH 7.2) contained 1 mm EDTA, 0.1 mM DTT, 2 mM 1-chloro-2,4-dinitrobenzene and 0.1 mM reduced glutathione. The enzyme source was 100 µL of supernatant from 10 larvae homogenized in 0.5 mL of phosphate buffer and centrifuged for 10 min at 10 000 g. The change in absorbance was measured at 340 nm for 2 min and converted to activity using the published extinction coefficient for this reaction and the estimated protein content of the supernatant measured by the method of Bradford (1976).

Table 1. Calculated LC_{50}, LC_{95} and RR_{95} values for four strains of *Aedes aegypti* as fourth-instar larvae after 24 h exposure to permethrin or DDT.

	Permethrin (mg/L)		DDT (mg/L)	
	LC_{50}	LC_{95} (RR_{95})	LC_{50}	LC_{95} (RR_{95})
Bora	0.00071	0.00152	0.106	0.263
Belem	0.003	0.013 (8.6)	1.11	14.22 (54)
Long Hoa	0.055	0.257 (169)	20.5	272.3 (1035)
Semarang	0.042	0.45 (296)	Plateau from 0.2–5	Around 45 (~171)

Esterase activity

The reaction was undertaken in phosphate buffered saline (PBS) (pH 6.5) containing 1% Triton-100, containing 0.2 mM α- or β-naphthyl acetate. Five larvae were homogenized in 0.25 mL of PBS and 25 μL of the homogenate was added to 125 μL of substrate solution. After 30 min at 25°C the reaction was stopped by the addition of 100 μL of Fast Garnett stain solution (8 mg Fast Garnett in 10 mL distilled water). Absorbance values were converted to nmol naphthol produced/min/mg protein using naphthol standard curves and protein values calculated from analysis of the insect homogenate as above.

Monooxygenase quantification

Buffer solution was as for the GST assay. A substrate solution of 7-ethoxycoumarine (7EC) (20 mM) was made in ethanol and 20 μL of this added to 1 mL of buffer. A single mosquito larva homogenate was combined with 100 μL of substrate and the conversion of 7EC to 7-hydroxycoumarine was measured at 450 nm after incubation for 4 h at 30°C. The reaction was stopped by the addition of 140 μL of glycine buffer (pH 10.4, 0.1 mM) before absorbance was measured.

Molecular and electrophysiological analysis of sodium channel mutations

RNA was isolated from samples of up to 10 individual mosquitoes from each strain and RT-PCR reactions were performed as previously described (Martinez-Torres *et al*., 1998) to amplify a partial (407 bp) sodium channel cDNA sequence (GenBank accession number AF534112). Purified PCR products were either directly sequenced, or subcloned into pGem T-easy vector (Promega, Southampton, UK) and sequenced. PCR products were sequenced in both directions with slightly nested primers AegF1 5′-AACTTACTCATTTCCATCATGG-3′ and AegRev 5′-TGCCGACAGCGAGGATGAACC-3′ using the ABI prism dye terminator cycle sequencing kit (ABI, Perkin-Elmer, Zarentrum, Belgium). Sub-cloned PCR products were sequenced with M13 primers. Alignments of the sequences and predicted translated peptide fragments were generated using the software package DNASTAR.

Electrophysiological studies were undertaken on dorsal nerve preparations of fourth-instar larvae of the susceptible Bora and pyrethroid-resistant Belem, Semarang and Long Hoa strains. The resistant strains were selected with permethrin at the 90% mortality level as one-day-old adults. F1 larvae from the survivors of this treatment were used for the electrophysiology studies. Dissected nerve preparations were checked for normal activity, then infused with increasing stepwise concentrations of permethrin or lambda cyhalothrin in normal saline as described by Hemingway *et al*., (1989) until a rapid firing response was observed. At least six individual larval preparations were tested for each strain.

Results

Fourth-instar larvae of all strains, with the exceptions of those from Bora, Bouaké and Larentuka, were resistant to permethrin. The highest level of resistance (296-fold at the LC_{95}) occurred in the Semarang strain (Tables 1 and 2). Resistance to DDT was present in larvae of the Belem, Long Hoa and Semarang strains compared to the pyrethroid-susceptible Bora strain (Table 2), the highest levels (~1000-fold) occurring in the Long Hoa strain. Calculation of accurate LC values for the Semarang strain was not possible due to the shallow slope with a plateau between 0.2 and 5 mg/L. There were also survivors of adults from the Long Hoa, Belem and Semarang strains after exposure

Table 2. Resistance ratios for permethrin at the LC_{95} in fourth-instar larvae of *Aedes aegypti* from a range of field collections and the frequency of sodium channel mutations found from a sample of up to 10 insects from each of these strains.

		Mutations		
Strain	RR_{95}	SS	RS	RR
Bora susceptible (Polynesia)	1	10	0	0
Paea (Polynesia)	4.4	1	0	0
Bouaké (Ivory Coast)	0.8	9	0	0
Baniona (Indonesia)	2.2	10	0	0
Larentuka (Indonesia)	1.3	10	0	0
Salatiga (Indonesia)	6.9	1	0	0
Semarang (Indonesia)	296	1	2	7
Mae Kud (Thailand)	5.4	1	0	0
Mae Kaza (Thailand)	7	8	1	0
Long Hoa (Vietnam)	169	0	0	10
Guyane (Cayenne)	20	2	5	2
Belem (Brazil)	8.6	0	3	6
Marlebel (Martinique)	35	5	2	2

Table 3. Percentage mortalities after exposure of adult *Aedes aegypti* to the WHO discriminating dosage of 0.1% lambda cyhalothrin.

Strain	% Mortality (sample size)
AEAE	100 (80)
Bora	100 (95)
Belem	38 (80)
Long Hoa	46 (80)
Semarang	62 (80)

to the WHO discriminating dosage of 0.1% lambda cyhalothrin, which killed 100% of the permethrin-susceptible Bora and AEAE strains (Table 3).

Biochemical analysis

Mean activity values (±SEM) were determined for 10 *Ae. aegypti* strains for GSTs, esterases and monooxygenases. Figure 1 shows that only the Guyane strain had substantially higher mean GST activity levels than those in the insecticide-susceptible Bora strain. Production of 7-hydroxycoumarine via monooxygenases was higher in the Salatiga and Paea strains than in the Bora strain, and significantly lower than the Bora strain in the Mae Kaza and Mae Kud strains from Thailand; all other strains had levels similar to that of Bora. Esterase activity was significantly higher than the Bora strain with α-naphthyl acetate in all strains except Long Hoa, but substantially higher only in the Salatiga, Semarang and Guyane strains with β-naphthyl acetate (Fig. 1).

Sodium channel analysis

A 407-bp cDNA sequence of the S6 hydrophobic segment of domain II of the sodium channel gene was amplified from up to 10 individuals from each of the 13 *Aedes* strains. Three silent polymorphisms were identified at nucleotide

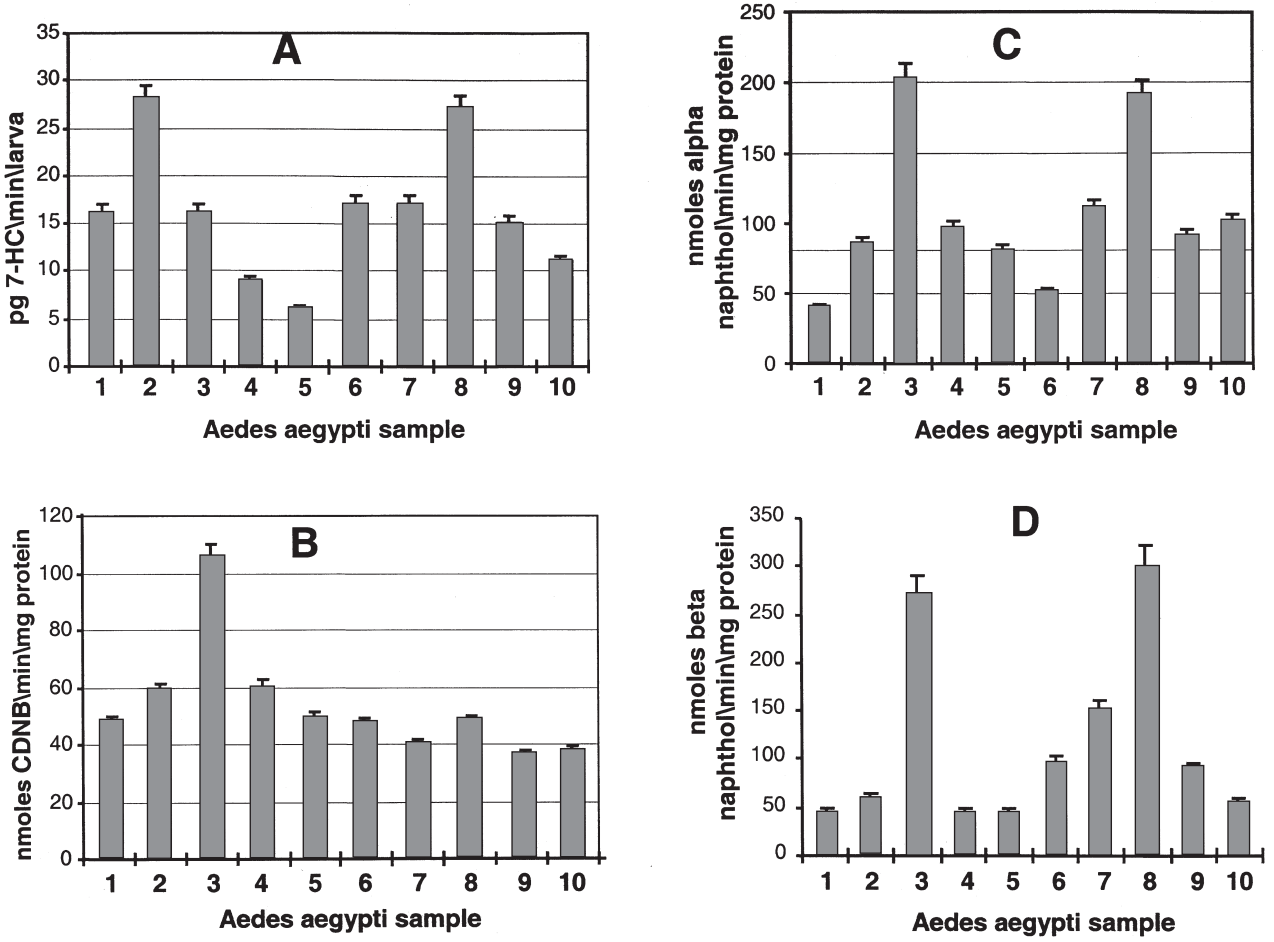

Fig. 1. Mean and standard deviations of monooxygenase (A), glutathione S-transferase (B), and esterase (C, D) activities in 10 strains of *Aedes aegypti*. Strains are arranged in geographical order by longitude from west to east: Bora (1), Paea (2), Guyane (3), Mae Kaza (4), Mae Kud (5), Long Hoa (6), Semarang (7), Salatiga (8), Larentuka (9) and Baniona (10).

	1										10										20										30										40									
***An. gambiae* RR**	-	-	-	L	N	L	L	I	S	I	M	G	R	T	M	G	A	L	G	N	L	T	F	V	L	C	I	I	I	F	I	F	A	V	M	G	M	Q	L	F	G	K	N
***Ae. aegypti* strains:**																																															
Baniona/Larentuka	W	P	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Belem/Guy/Marl	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**V**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Long Hoa	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Mae Kaza/Semarang	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

										50										60										70										80									
***An. gambiae* RR**	Y	V	D	N	V	D	R	F	P	D	H	D	L	P	R	W	N	F	T	D	F	M	H	S	F	M	I	V	F	R	V	L	C	G	E	W	I	E	S	M	W	D	C
***Ae. aegypti* strains:**																																															
Baniona/Larentuka	*	**I**	*	*	*	*	*	*	*	*	**K**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Belem/Guy/Marl	*	**I**	*	*	*	*	*	*	*	*	**K**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Long Hoa	*	**I**	*	*	*	*	*	*	*	*	**K**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**W**	*	*	*	*	*	*	*	*	*	*	*	*
Mae Kaza/Semarang	*	**I**	*	*	*	*	*	*	*	*	**K**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

										90										100										110										120									
***An. gambiae* RR**	M	L	V	G	D	V	S	C	I	P	F	F	L	A	T	V	V	I	G	N	F[1]	V	V	L	N	L	F	L	A	L	L	L	S	N	F	G	S	S	S	L	S	A	P
***Ae. aegypti* strains:**																																															
Baniona/Larentuka	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**L**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Belem/Guy/Marl	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**M**	*	*	**L**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Long Hoa	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**L**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Mae Kaza/Semarang	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**L**	*	**G**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

[1] leucine →phenylalanine mutation found in the West African pyrethroid resistant strain of *Anopheles gambiae*

Fig. 2. Sequence analysis of domain II of the voltage-gated sodium channel in the susceptible Larentuka and Baniona strains of *Aedes aegypti* compared to the permethrin-resistant strains Belem, Guyane (Guy), Marlebel (Marl), Semarang, Mae Kaza and Long Hoa. The putative amino acid changes are highlighted and compared to the available sequence in pyrethroid-resistant *Anopheles gambiae* RR from West Africa (GenBank accession number CAA73920) (Martinez-Torres *et al.*, 1998).

positions, 164 (A/G), 194 (C/T) and 227 (A/G). These polymorphisms were very common in all strains regardless of whether there were substitutions resulting in possible resistance-associated amino acid changes present. Samples of 10 mosquitoes from each of the Bora, Bouaké, Baniona and Larentuka strains had sequences in the S6 segment that were identical to the wild type susceptible AEAE sequence. This correlates well with the permethrin susceptibility status of these strains, the Baniona strain showing only two-fold resistance to permethrin and the other strains being fully susceptible. An alignment of the putative amino acid sequences for all strains compared to the resistant West African *An. gambiae* sequence for *kdr* (Martinez-Torres *et al*., 1998) is given in Fig. 2. Six pyrethroid-resistant *Aedes* strains had nucleotide substitutions that would result in predicted amino acid changes within the S6 segment of domain II compared to the wild type, although none involved the classic Leu → Phe substitution seen in other pyrethroid resistant insects. All insects from Long Hoa (Vietnam) were homozygous for a leucine to tryptophan substitution at amino acid position 75 as a result of the double nucleotide change TTA to TGG. One of these nucleotide changes is at the same site as the silent polymorphism, reported above, at nucleotide position 227.

All samples from Belem (Brazil) were either homozygous or heterozygous for a double amino acid change. This involved an isoleucine to methionine substitution at amino acid position 104, due to an A/G mutation at nucleotide 314, and a glycine to valine substitution at amino acid 16, due to a G/T mutation at nucleotide 49. Three mosquitoes were heterozygous for both these mutations, whereas the other six were homozygous for both mutations. *Aedes aegypti* from Marlebel (Martinique) and Guyane had identical mutations to those from Belem. In Marlebel, there were five wild type (SS) individuals, two mosquitoes homozygous for both mutations (RR) and two heterozygous (RS) for both mutations. In the Guyane strain, numbers were 2RR, 5RS and 2SS.

Samples from Semarang (7RR, 2RS and 1SS) (Indonesia), and Mae Kaza 1RS (Thailand), shared a mutation at amino acid position 109, involving a valine to glycine substitution from a single T to G nucleotide change at position 329 (Table 2). In addition to these specific mutations between the *Ae. aegypti* strains, there were two non-silent polymorphisms between the *An. gambiae* and *Ae. aegypti* sequences at amino acid positions 45 and 54. Both substitutions were conservative changes (Fig. 2), and both are also seen in the orthologous sequence from *Cx. quinquefasciatus* (GenBank accession number CAB38171, submitted by Martinez-Torres *et al*., 1998).

To determine whether any of the observed mutations resulted in a change in sensitivity to pyrethroids, direct neurophysiological measurements were made on the exposed dorsal nerve cord of fourth-instar larvae. The Long Hoa and Belem strains required significantly higher concentrations of both permethrin (~29-fold and ~1400-fold, respectively) and lambda cyhalothrin (~15-fold and ~740-fold, respectively) to induce repetitive firing than the susceptible strains. In contrast, the Semarang strain was almost as susceptible to lambda cyhalothrin as the Bora and AEAE strains, requiring only marginally (~3-fold) more insecticide to induce repetitive firing, but was permethrin resistant (~14-fold more insecticide required) (Table 4). Subsequent PCR analysis of the carcasses used for the neurophysiological determination showed all six individuals from Belem and Semarang to be homozygous for the mutant phenotype, suggesting that insecticide selection had positively selected these genotypes. The Long Hoa strain was not analysed by PCR, being fixed for the mutant allele.

Table 4. Pyrethroid concentrations at which repetitive firing of a fourth-instar larval dorsal nerve cord preparation occurred after electrical stimulation. Values for all strains are the means of at least five different larval preparations.

	Insecticide concentration (M)	
Strain	Permethrin	Lambda cyhalothrin
Bora	6×10^{-10}	7.0×10^{-11}
AEAE	1×10^{-10}	6.4×10^{-11}
Belem	5×10^{-7}	5.0×10^{-8}
Long Hoa	1×10^{-8}	1.0×10^{-9}
Semarang	5×10^{-9}	2.0×10^{-10}

Discussion

Of 13 field-collected *Ae. aegypti* populations from seven countries in Asia, Latin America and Africa, 11 were resistant to permethrin, confirming earlier suggestions (Malcolm, 1988) that pyrethroid resistance is common in this species. Metabolic detoxification mechanisms of resistance (esterase, glutathione-S-transferase and/or monooxygenase-based) are implicated for several of these strains (Salatiga, Paea, Mae Kud, Mae Kaza, Guyane and Semarang). The presence of resistance to DDT in three permethrin-resistant strains (Guyane, Semarang and Belem), without elevated GST activity in the latter two, also suggested the involvement of a target-site resistance mechanism. Many reports of pyrethroid resistance predate the use of pyrethroids for *Aedes* control in the field (Rongsriyam & Busvine, 1975), hence some of this pyrethroid resistance may be a legacy of cross-resistance from earlier or current use of DDT for indoor residual spraying to combat malaria. Comparable ratios of resistance for permethrin and DDT supported this cross-resistance hypothesis, although in some cases the DDT resistance ratio was higher than that for permethrin. PCR analysis targeting the domain of the sodium channel in which *kdr* mutations have previously been documented was therefore undertaken.

We have identified four different amino acid substitutions in the S6 hydrophobic segment of domain II of the *Ae. aegypti* sodium channel gene in pyrethroid resistant mosquitoes, which differ from the laboratory wild type and recently field-caught pyrethroid-susceptible strains. The classic Leu → Phe mutation found in a range of different insects, including the mosquito *An. gambiae*, was

not observed in any of the *Aedes* strains, probably because the nucleotide codon bias in *Ae. aegypti* will not allow this amino acid substitution via mutation of a single nucleotide. One of the amino acid substitutions found in the Long Hoa pyrethroid-resistant *Ae. aegypti* from Vietnam involves a double nucleotide change from the susceptible sequence, but this is at the same site as a common silent polymorphism found in a range of our *Ae. aegypti* strains, which accounts for one of the changes.

Different mutations of the sodium channel gene have been seen in other pyrethroid-resistant insects. For example, West African *An. gambiae* has a Leu → Ser substitution rather than Leu → Phe (Ranson *et al.*, 2000), and 12 other amino acid changes have been found in the 538 ge strain of housefly compared to the wild type sequence, none of which involve leucine[1014] (Ingles *et al.*, 1996). In field-selected resistant strains analysed to date, mutations tend to be concentrated in a single hydrophobic region of one domain of this large protein (Jamroz *et al.*, 1998). However laboratory selections of *Drosophila* temperature-sensitive mutants, field-collected pyrethroid-resistant German cockroach strains and *Heliothis* field pyrethroid-resistant strains suggest that mutations in other domains with similar phenotypes are possible (Park & Taylor, 1997; ffrench-Constant *et al.*, 1998; Liu *et al.*, 2000). Our results, although identifying novel mutations in this domain associated with resistance, do not preclude the presence of other important mutations elsewhere in the coding region of the *Ae. aegypti* sodium channel gene.

Some but not all of the mutations described here in domain II of the sodium channel gene are associated with significantly different levels of nerve sensitivity to permethrin and lambda cyhalothrin inhibition. The single nucleotide substitution at position 75 (Long Hoa) and the double substitution at positions 104 and 16 (Belem) are associated with large changes in both permethrin and lambda cyhalothrin sensitivity compared to the susceptible strains. The Semarang strain with the substitution at position 95 requires only marginally more (~three-fold) lambda cyhalothrin to induce repetitive nerve firing than the susceptible strains, but is slightly more insensitive to permethrin. All three strains demonstrate more sensitivity to lambda cyhalothrin than to permethrin. Neurophysiological studies on *Cx. quinquefasciatus* from Saudi Arabia indicated that *kdr*-type nerve insensitivity in this species, unlike houseflies, might not confer resistance to all pyrethroids (Amin & Hemingway, 1989).

It is of interest that, whereas three strains (Belem, Marlebel and Guyane) share the double substitution at 104 and 16, only Belem had detectable DDT resistance. One hypothesis for this would be that seven out of nine individuals sequenced for both Marlebel and Guyane had at least one wild-type allele, whereas this was true for only three out of nine individuals from Belem. It is possible that DDT resistance is strongest in individuals homozygous for *kdr* mutations. Both the other two DDT-resistant strains, Long Hoa and Semarang, also had high proportions of *kdr* homozygotes (ten out of ten and seven out of ten, respectively). Permethrin resistance does not seem to be similarly related, as both Guyane and Marlebel had higher permethrin resistance ratios than Belem.

The relative contributions of metabolic detoxification and target-site mechanisms of resistance are strain-dependent. For example, the Long Hoa strain is highly resistant to both permethrin and DDT, and it appears from the data presented that this may be due solely to a *kdr* mutation, as no alterations in enzyme detoxification activities were detected. In contrast, the Semarang strain has both a *kdr* mutation and elevated esterase levels, and the Guyane strain has elevated GST and esterase levels as well as two *kdr* mutations. Different patterns of cross-resistance have implications for resistance management programmes involving multiple pyrethroids, and underline the need for understanding the types and combinations of insecticide resistance mechanism present in field strains.

Recent cloning of the para-voltage-gated sodium channel gene has allowed the development of PCR-based assays for detection of the nerve insensitivity *kdr*-type resistance, which results from DDT/pyrethroid cross-resistance. Identification of mutations causing resistance is an essential first step in this process. Different PCR diagnostic assays for resistant East and West African *An. gambiae* with the Leu → Phe or Leu → Ser substitutions have been published (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). Our current results suggest sequencing of those sodium channel gene regions in which mutations conferring changes in pyrethroid sensitivity occur is essential when looking initially at any new resistant strain of *Ae. aegypti*, as applying 'diagnostic' assays prior to this may lead to false negative results.

Molecular data on the major organophosphorus insecticide resistance mechanisms in *Cx. quinquefasciatus* suggest that resistance may be a rare event, with migration playing a significant role in the world-wide spread of a small number of initial molecular changes that produce resistance (Raymond *et al.*, 1991; Hemingway & Karunaratne, 1998). If migration is a common mechanism for the rapid spread of resistance, this has important implications for insecticide resistance management programmes. *Aedes* mosquitoes are unusual in that their eggs, unlike those of *Anopheles* and *Culex*, can survive desiccation. *Aedes* mosquitoes often breed in close association with man, resulting in their inadvertent spread, for example, in water containers or as car tyres are transported, often in the form of dried eggs. This effective dispersal mechanism could have contributed to the spread of pyrethroid resistance in *Ae. aegypti*, potentially generating homogeneity of resistance in different geographical areas at levels similar or greater to those of resistance in *Cx. quinquefasciatus*. The tight linkage of two mutations in the sodium channel gene of strains of *Ae. aegypti* from Martinique, French Guiana and Brazil suggest that pyrethroid resistance in these strains has a common origin. However, the polymorphism in DNA sequence in our *Ae. aegypti* data suggests that target site-based pyrethroid resistance in this species has multiple origins and must have arisen at least three times through different mutations, despite the high level of mobility of this species.

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