INSECTICIDE RESISTANCE AND THE VECTORIAL CAPACITY OF *CULEX QUINQUEFASCIATUS*, THE VECTOR OF FILARIASIS

S.H.P.P. KARUNARATNE and J. HEMINGWAY*
Department of Zoology, University of Peradeniya, Peradeniya and
**School of Biosciences, Cardiff University, Wales, UK.*

ABSTRACT

Filariasis, caused by the nematode parasite *Wuchereria bancrofti* is endemic in Sri Lanka. The mosquito *Culex quinquefasciatus* is the vector of filariasis in Sri Lanka and other tropical countries. Control of this mosquito is mainly by organophosphorus insecticides. Resistance mechanism of *Culex* mosquitoes to organophosphorous insecticides is the elevation (increased quantities) of mosquito carboxylesterases and occurs in more than 80% of resistant *Culex* worldwide. Biochemical characterisation of these carboxylesterases, purified from *C. quinquefasciatus* from Colombo, Sri Lanka, revealed that they bind and inactivate organophosphorous insecticides very fast. Antiserum was raised against these esterases and respective genes were isolated. It was found that gene amplification was responsible for the increased production and the resistant insects have upto 80 copies of the gene. Electron-microscopic studies, using mosquito tissues and the antiserum, revealed that the enzymes in resistant insects are expressed at very high levels in the mosquito gut and sub-cuticular layer. As the parasites must pass through some of these tissues to complete their development, parasite survival and hence vectorial capacity can be affected by the insecticide resistance status of the insect. However, insecticide resistance is assumed to increase the likelihood of disease transmission by increasing the population size and allowing the mosquitoes to live longer in the presence of insecticides. This hypothesis was tested for the first time.

Blood fed female *C. quinquefasciatus* were collected from seven filariasis endemic areas in Sri Lanka. Mosquitoes were individually analysed for parasite loads by quantitative PCR and insecticide resistance by biochemical assay with para-nitrophenyl acetate (pNPA). Almost 80% of the mosquitoes were infected with *W. bancrofti*. However, there was a strong negative correlation between esterase activity levels with pNPA and parasite RNA levels in all seven localities. Membrane feeding of the insecticide resistant and susceptible mosquito colonies with *W. bancrofti* infected blood produced infective parasite larvae after twelve days in 76% of the susceptible, and none of the resistant females. Results suggest that elevated esterase activity affects the development of *W. bancrofti* larvae which may be arrested in the gut cells of insecticide resistant, but not susceptible mosquitoes. Filarial worm infection of mosquitoes cause significant damage to the mosquito host, and high microfilaria infections result in insect mortality. The predominance of the elevated esterase-based resistance mechanism in field populations of *C. quinquefasciatus* may therefore be influenced by the dual roles of insecticide detoxication and reduction of microfilarial burdens.

INTRODUCTION

Filariasis is endemic to Sri Lanka. The endemic zone is mainly the western and southern coastal belt, extending from Puttalam to Katharagama. In addition, there is an endemic patch in Kurunegala District. Filariasis is caused by the nematode parasite *Wuchereria bancrofti* and

transmitted from man to man by the mosquito vector *Culex quinquefasciatus*. Control of this mosquito is mainly by the use of organophosphorus insecticides. The most commonly observed change that has been linked to resistance development is the development of increased activity of mosquito carboxylesterases that can be easily detected by native polyacrylamide gel electrophoresis (native PAGE) with α- and β- naphthylacetate as the substrate (Karunaratne, 1998). This resistance mechanism occurs in more than 80% of resistant *Culex* worldwide (Raymond *et al.* 1991). Increased activity of carboxylesterases is due to the presence of increased (elevated) quantities of the enzyme in insecticide resistant mosquitoes. It was found that gene amplification was responsible for the increased production and the resistant insects have upto 80 copies of the gene (Vaughan and Hemingway, 1995).

Mosquito carboxylesterases were purified from insecticide resistant mosquitoes. Biochemical characterization of purified enzymes were carried out to determine its role in insecticide resistance. Distribution of these enzymes within the mosquito body was determined using antisera raised against these enzymes. Effect of the presence of increased carboxylesterase amounts on the parasite survival and hence on the vectorial capacity was tested for the first time.

MATERIALS AND METHODS

Mosquitoes

Insecticide susceptible PelSS and resistant PelRR strains were derived from *C. quinquefasciatus* mosquitoes collected from Peliyagoda. For parasitic DNA studies, mosquitoes were collected from seven filariasis endemic areas namely Kalubowila, Maharagama, Nugegoda, Ratmalana, Kalutara, Kurunegala and Polgahawela. Blood fed female *C. quinquefasciatus* were collected from houses and snap frozen <48 hrs after taking their blood meal.

Enzyme purification

Carboxylesterase activities were assayed with the substrate para-nitrophenyl acetate (pNPA) (1mM) in 50 mM phosphate buffer (pH 7.5) at 22^{0}C. The protein concentration was estimated using bovine serum albumin as the standard protein (Karunaratne et al. 1993; 1995). Batches of 4th instar larvae were homogenized and centrifuged at 15,000g for 5 min, and the supernatant was taken. Carboxylesterases were then purified by sequential column chromatography using Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow, Hydroxylapatite, para-chloromercuribenzoate and preparative electrophoresis (K93,95). Final enzyme preparations were homogeneous as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Kinetic constants

For the inhibition kinetics, stopped time inhibition assays were performed. Insecticide stock solutions were prepared in acetonitrile and diluted in phosphate buffer (pH 7.4). Purified enzyme was incubated with a series of concentrations of the test insecticide [acetonitrile concentration never exceeded 1% (v/v)] for fixed time intervals. Residual activity was determined from the rate of substrate hydrolysis. Inhibitor concentrations were usually in large excess so that linear pseudo-first order kinetics were obtained. The bimolecular rate constants for the formation of the acylated enzyme (k_a) were derived. If inhibitor concentration could

not be maintained in large excess, k_a values were determined in the presence of substrate (Karunaratne *et al.* 1993; 1995).

Reactivation experiments were performed by incubating the purified enzyme with the insecticide for 10 - 15 min, so that the enzyme was >90% inhibited. The unbound insecticide and enzyme-insecticide complex were separated on Nick spin columns. Aliquots of the enzyme were then removed over a 12 hr time course to measure the residual activity. The slope of the curve, obtained by plotting the percentage remaining activity against time, gave the reactivation constant k_3.

Polyclonal antiserum was raised against purified native enzyme in a rabbit by standard methods. Cross-reactivity to different mosquito tissues was tested using horseradish peroxidase-labelled secondary antibody. The bound antibody was detected by Enhanced Chemo-Luminescent (ECL) method using an electron microscope (Karunaratne, Jayawardena and Hemingway, 1995).

Determination of the amount of parasitic cDNA

Individual mosquitoes were homogenized in 100 µl ice-cold phosphate buffer (50 mM, pH 7.4), which had previously been treated with diethyl pyrocarbonate (DEPC) in order to inhibit RNase activity. The homogenate was centrifuged at 12,000g, 4°C for 5 min and the supernatant was removed. 30 µl of the supernatant was used for the esterase assay. Remaining supernatant was used to extract total RNA. Each homogenate was mixed with TRI reagent (Sigma) and the mixture incubated at room temperature for 5 min. After extraction with chloroform, RNA was precipitated using isopropanol. All reagents for cDNA synthesis were obtained from Gibco BRL. 10 µl of RNA was incubated with 0.5 µg oligo $(dT)_{15}$ (Promega) at 70°C for 10 min. cDNA synthesis mixture (1 x first strand buffer, 7.14 mM DTT, 0.36 mM dNTPs) was added. Reaction mixtures were incubated for 2 min at 42°C; 200 Units of superscript RT were added and incubation continued for a further 50 min. The reaction was terminated by heating at 70°C for 15 min. Quantitative PCR was carried out on a Roche Light Cycler. PCR reaction mixtures comprised 1 µl template cDNA, 0.1 mM each primer, 1 x reaction buffer (containing SYBR Green, Taq DNA polymerase, dNTP mix, 10 mM $MgCl_2$). PCR primers were obtained from Sigma Genosis. Quantitative PCR used 40 cycles of 95°C for 1 second, 55°C for 2 seconds and, 72°C for 10 seconds. Data were acquired at the end of each cycle by measuring fluorescence at 80°C. Results were standardised using an internal mosquito actin control for each sample.

RESULTS

Multiple preparations routinely gave approximately 40% final recovery of enzyme activity and 50-350 fold purification of carboxylesterases. The relative monomeric mass of the purified enzyme, estimated from SDS-PAGE, was 62,000 ± 2400. The pI of 5.0 was determined by iso-electric focusing on Ampholine PAGE plates.

Enzyme inhibition kinetics showed that for organophosphates, the bimolecular rate constants (k_as) were very high and deacylation rates (k_3s) were very low (*eg.* for malaoxon, k_a and k_3 values were $5.0 \pm 0.17 \times 10^4$ M^{-1} min^{-1} and $4.3 \pm 0.11 \times 10^{-4}$ respectively). This shows that the main role of these enzymes is sequestration *ie.* rapid binding followed by slow turn over (Karunaratne *et al.* 1993; 1995).

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Cross-reactivity studies with antiserum and different mosquito body tissues showed that these carboxylesterases are expressed at very high levels in the mosquito gut, sub-cuticular layer, malpighian tubules and salivary glands in resistant mosquitoes (Hemingway and Karunaratne, 1998).

Almost 80% of both field collected insecticide susceptible and resistant mosquitoes were infected with *W. bancrofti*. However, there was a strong negative correlation between esterase activity levels with pNPA and parasite RNA levels in all seven localities (McCarroll *et al.* 2000). Membrane feeding of the insecticide resistant PelRR and susceptible PelSS mosquito colonies with *W. bancrofti* infected blood, with intermediate levels of parasitemia, which should result in infected mosquitoes without substantial insect mortality, produced L3 infective parasite larvae after 12 days in 76% of the PelSS females (n=250) but none of the PelRR (n=200).

DISCUSSION

Characterization of purified mosquito carboxylesterases showed that the role of these enzymes in insecticide resistance was sequestration *ie.* rapid binding followed by very slow deacylation or turn over. This means that in the presence of large quantities of enzymes they can absorb (or sequester) a large amount of insecticides within a remarkably short time. Therefore, the resistant individuals have to produce a considerable amount of these enzymes to maintain the system effectively. Gene amplification was shown to be the cause for the increased production. It is surprising that such a mechanism, which results in considerable energy loss to the insect, has been selected. However, there is no evidence to show that the resistant *Culex* individuals with elevated esterases are less fit in their diurnal activities than the susceptible individuals with no elevated esterases.

It is interesting to note that the pattern of carboxylesterase distribution within the mosquito body is such that it prevents the entry of insecticides into the body. Insecticide molecules which enter through the cuticle are absorbed and detoxified by the carboxylesterases accumulated just beneath the cuticle. Those which enter through the food are partly inactivated by high carboxylesterase quantity in mosquito saliva. Carboxylesterases in gut epithelium act to prevent the entry of insecticide molecules into the body cavity from the gut lumen.

As the *W. bancrofti* parasites must pass through the mosquito gut epithelium to complete their development, parasite survival and hence vectorial capacity can be affected by the insecticide resistance status of the insect. However, insecticide resistance is assumed to increase the likelihood of disease transmission by increasing the population size and allowing the mosquitoes to live longer in the presence of insecticides. This hypothesis was tested for the first time.

Our results suggest that elevated esterase activity affects the development of L1 *W. bancrofti* larvae which may be arrested in the gut cells of insecticide resistant, but not in susceptible mosquitoes. Filarial worm infection of mosquitoes cause damage to the mosquito host, and high microfilaria infections result in insect mortality. The predominance of the elevated esterase-based resistance mechanism in field populations of *C. quinquefasciatus* may therefore be influenced by the duel roles of insecticide detoxication and reduction of microfilarial burdens. Similar elevated esterase-based resistance mechanisms have recently

been found in Sri Lankan field populations of the malaria vectors *Anopheles culicifacies* and *An. subpictus* (Karunaratne, 1999). Unlike *W. bancrofti* larvae, malaria parasites have to pass also through mosquito salivary glands to complete their life cycle. Therefore, if the pattern of carboxylesterase distribution in *Anopheles* vectors is also the same, it may directly affect malaria transmission.

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