Short Report: Pyrethroid Susceptibility in Natural Populations of the *Anopheles punctulatus* Group (Diptera: Culicidae) in Papua New Guinea

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Abstract. The development of insecticide resistance has compromised mosquito control efforts in many parts of the world. Papua New Guinea (PNG) has a long history of dichlorodiphenyltrichloroethane (DDT) use and currently distributes pyrethroid-treated nets for malaria control. This study is the first to investigate the status of pyrethroid resistance in the *Anopheles punctulatus* group, the major malaria and filariasis vectors of PNG. The study used World Health Organization standard susceptibility bioassays to detect knockdown phenotypes and a novel nested polymerase chain reaction to detect the knockdown resistant (*kdr*) allele in these vectors. Our results show 100% susceptibility to pyrethroids in all populations surveyed and an absence of the *kdr* allele.

INTRODUCTION

The use of dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides against mosquitoes is an important way to control transmission of human disease. However, prolonged and intensive use of these agents may result in the development of insecticide resistance in exposed mosquito populations, as reported in many parts of the world, and threatens success of pyrethroid-based vector control programs.^{1–3}

The primary mechanism of resistance to DDT and pyrethroids is the knockdown resistance (*kdr*) allele,^{1,4} a point mutation in the voltage-gated sodium channel gene (*vgsc*). This mutation results in the substitution of leucine for phenylalanine at amino acid residue 1014 (L1014F).^{1,4} Because DDT and pyrethroids share a common mode of action, development of resistance resulting from the use of either insecticide can cause cross-resistance to the other.^{2,4}

Malaria is holoendemic in the lowlands of Papua New Guinea (PNG) and is one of the leading causes of morbidity and mortality. *Plasmodium falciparum* and *P. vivax* are the most common species, although *P. malariae* and *P. ovale* are also present in some areas.⁵ Mosquitoes within the *Anopheles punctulatus* group are the primary vectors of both malaria and filariasis in PNG. This group comprises at least 12 species, among which *An. punctulatus* sensu stricto (s.s.), *An. farauti* s.s., *An. hinesorum* (previously *An. farauti* 2), *An. farauti* 4, and *An. koliensis* are abundant with distinct geographic distributions.⁶

From the late 1950s through the 1970s, a DDT residual spray program was conducted intensively in many parts of PNG for the control of malaria.⁷ In the early stages of the spray campaign, World Health Organization (WHO) susceptibility tests revealed very high levels of susceptibility to DDT in the *An. punctulatus* group in many parts of PNG and Solomon Islands.^{8,9} However, the program ceased after operational failure; malaria transmission remained high because of a change in feeding behavior of the vector.¹⁰ There were no follow-up reports on the susceptibility status. From the early 1990s to the present, pyrethroid-treated bed nets have been distributed

in many parts of the country (unpublished data). Recently, there has been an extensive distribution of long-lasting insecticide-treated nets (LLIN) throughout PNG by the National Department of Health (NDOH) and Rotary Against Malaria (RAM). Despite extensive use of insecticides, the status of resistance to pyrethroids in PNG has never been determined.

The objective of this study was to determine the status of knockdown resistance to pyrethroids in the *An. punctulatus* group in PNG. We hypothesized that resistance may be present in some populations of PNG as a result of DDT use in the past. Because the Global Fund is supporting deployment of LLIN in PNG, it is important to provide baseline data on the current prevalence of knockdown resistance to monitor its development in the future.

We sampled five populations of the *An. punctulatus* group in three malaria- and filariasis-endemic provinces of PNG, each with different histories of insecticide coverage, intensity, and application methods. These sites were chosen in an effort to identify resistance that may be present as a result of various levels of exposure. The populations Usino-Bundi and North Coast (Madang Province) have a brief history of DDT indoor residual spray and recent distribution of LLIN. Drekikir (East Sepik Province) has a history of DDT spraying in the past and recent distribution of LLIN.⁷ Lorengau (Manus Province) not only has a long history of DDT spray⁷ but a long history of LLIN use as well, with extensive distribution (William Popon, Disease Control Officer, Manus Provincial Health Department, personal communication). Ramu Sugar Plantation (Madang Province) has been spraying pyrethroids in agricultural areas consistently over three decades.^{11,12} In addition, indoor residual spraying of community houses using lambda-cyhalothrin is practiced annually in the area (Lastus Kuniata, Ramu Entomology Department, personal communication).

Larvae of all stages were collected from all visible breeding sites in each population. The larvae were reared into adults, and 2- to 5-day-old females were tested for the knockdown resistant phenotype using two WHO insecticide susceptibility bioassays: tube assay with 0.05% lambda-cyhalothrin-treated filter paper and cone assays using a new 55 mg/m² deltamethrin-treated LLIN (produced by PermaNet).^{13–15} Control assays were conducted using filter paper treated with solvent only and an untreated bed net. To learn more about the subtle differences between populations, we included additional

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sampling intervals throughout the exposure or post-exposure period to determine when knockdown events occur. Kaplan–Meier survival curves were constructed, and log-rank tests were used to compare curves between populations. α values were modified using the Bonferroni correction for multiple comparisons. All statistics were completed in PASW Statistics 17.0.3 (IBM, Somers, NY).

Mosquitoes were morphologically identified,¹⁶ and genomic DNA was extracted for species confirmation and *kdr* detection. Polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer region 2 (ITS2)¹⁷ was done on the morphologically identical members of *An. farauti* s.l. to determine species.

A nested PCR was developed to detect *kdr* in *An. punctulatus*. A positive genomic control possessing the *kdr* mutation (TTT) was created by introducing a point mutation in a *VGSC* PCR representative sample using Stratagene QuikChange Site-Directed Mutagenesis Kit in accordance with the manufacturer's protocol. These samples were then sequenced to confirm the presence of the *kdr* point mutation.

The Nest 1 PCR involves the addition of genomic DNA to a reaction mixture (25 μ L final volume) containing primers modified from Martinez-Torres and others⁴: aDip1 (5'-TGGC CSACRCTGAATTTACTC-3') and cDip2 (5'-TTKGACAA AAGCAAGGCTAAGAA-3'). Amplification of the Nest 1 *kdr* target sequence was performed using the following thermocycling program: 95°C for 2 minutes (1 $\times$); 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 45 seconds (46 $\times$); and 72°C for 4 minutes (1 $\times$). The Nest 1 PCR product (10 μ L) was added to another reaction mixture (25 μ L final volume) containing primers N2kdrFWD812 (5'-GTAGAA GGTAAAMTTTTTCTTACT-3') and cDip2. Amplification of the Nest 2 *kdr* target sequence was performed using the same thermocycling program used to amplify the Nest 1 *kdr* target. The Nest 2 PCR product was digested with *DdeI* restriction enzyme at 37°C for 1 hour. The enzyme will induce a cut if the wild type is present and not if the *kdr* mutation is present. Gel electrophoresis was performed on the digestion products and their corresponding Nest 2 products. The *kdr*-negative homozygote shows a single digestion band at 551 bp as a result of the *DdeI* cutting. The *kdr*-positive homozygote shows an undigested band at 637 bp. The *kdr* heterozygote shows two bands at 551 and 637 bp.

The species composition from each study population (Table 1) varies greatly because of the differences in habitat preference and geographic distribution of each species of the group.⁶

For every population, 100% knockdown was observed within 60 minutes post-exposure to the deltamethrin-treated net, and 100% knockdown was also observed within 60 minutes

of exposure to the λ -cyhalothrin-treated paper. No recovery was observed in any population after 24 hours. According to the WHO standards, 98–100% mortality indicates insecticide susceptibility in the population.¹⁴

Subtle differences in survival were observed between populations. In the cone assay, estimated mean survival times ranged from 6.62 ± 0.46 minutes in Ramu to 21.55 ± 1.70 minutes in Usino-Bundi. The Usino-Bundi population exhibited significantly longer survival times than the others (log-rank test, $P < 0.001$) (Figure 1A). In the tube assay, estimated mean survival times ranged from 16.55 ± 0.69 minutes in Lorengau to 25.08 ± 0.81 minutes in North Coast. The North Coast population exhibited significantly longer survival times than the others (log-rank test, $P < 0.001$) (Figure 1B).

Interestingly, the two populations with the greatest insecticide exposure, Ramu and Lorengau, had the fastest times to knockdown. One difference separating these populations from the others is that they are comprised of significant numbers of *An. farauti* s.l. In Lorengau, only *An. farauti* s.s. was caught, whereas in Ramu, nearly one-half of the mosquitoes were *An. hinesorum*. All other populations were almost strictly *An. punctulatus*. Studies have shown that *An. farauti* s.l. developed behavioral resistance to avoid contact with DDT in the past.^{8,10,18} In addition, both *An. farauti* s.s. and

FIGURE 1. Kaplan–Meier curves showing time to knockdown for each population (A) after being exposed to 55 mg/m² deltamethrin-treated net and (B) during exposure to 0.05% λ -cyhalothrin-treated paper. Curves that do not contain the same letter, as indicated in the legend, are significantly different (P values < 0.001 , Bonferroni-corrected $\alpha = 0.003$).

TABLE 1
Anopheles species composition by collection site

Population	GPS coordinates	Total mosquitoes	Species composition		
			<i>An. farauti</i> s.s.	<i>An. hinesorum</i>	<i>An. punctulatus</i>
Ramu	145.981°E, 6.090°S	58	0	24	34
Usino-Bundi	145.550°E, 5.433°S	150	0	0	150
North Coast	145.627°E, 4.795°S	169	0	1	168
Drekikir	142.683°E, 3.571°S	300	0	0	300
Lorengau	147.262°E, 2.034°S	206	206	0	0

An. hinesorum are early exophilic biters, whereas *An. punctulatus* bites indoors in the very early morning.^{17,19} These behavioral differences may reduce the selection pressure on *An. farauti* s.s. and *An. hinesorum* to develop physiological resistance. Although mosquitoes in the Ramu valley have been under significant, sustained insecticide pressure for over the last three decades, susceptibility was likely preserved by the rotation of pesticides.¹¹

The *kdr* diagnostic was developed to aid in resistance monitoring; however, no mosquitoes were found with a resistant phenotype. As a result, the assay was applied to only a subset (25%) of the bioassayed mosquitoes to test the sensitivity of the assay for future use. As expected, all mosquitoes tested were wild type at the *kdr* locus. The development of this assay will prove useful in monitoring the impact of vector control on mosquito populations.

This study was conducted because of worldwide reports of the development of physiological resistance to DDT and pyrethroids in important mosquito vectors of human diseases as a result of intensive insecticide use.^{20–23} The long history of DDT use in malaria-endemic areas of PNG as well as pyrethroid use for agricultural and public health purposes justify the need to survey for resistance in PNG vectors. In light of the recent nationwide distribution of LLIN, this information will inform officials regarding the sustainability of such large-scale vector control programs.

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