

PAPUA NEW GUINEA & THE GLOBAL FUND
MALARIA CONTROL PROGRAM EVALUATION

**INSECTICIDE RESISTANCE MONITORING:
REPORT ON PHENOTYPIC RESISTANCE OF ANOPHELINES
TO DELTAMETHRIN, LAMBDA-CYHALOTHRIN AND DDT
(2016)**

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Executive Summary

Insecticide resistance monitoring is an important component of vector borne disease control. The last assessment of insecticide resistance in PNG was conducted in 2010, when no resistance was found. Since then, vector populations have been exposed to considerably higher levels of pyrethroids (deltamethrin) due to the successful nation-wide distribution of insecticide treated nets. In the present study, we provide an update on insecticide resistance in four traditionally highly malaria endemic areas.

Insecticide resistance against deltamethrin, lambda-cyhalothrin and DDT was assessed using WHO bioassays for phenotypic susceptibility testing of female anophelines. Over 100 (total 108) bioassays for each insecticide were conducted screening a total of 2,290 adult female anopheline mosquitoes. No phenotypic resistance was observed and the bioassay parameters agreed well with those observed in other studies using the same assay and insecticides.

In conclusion, the results of the present study indicate that the three tested insecticides are still universally effective in PNG, even though signs of at least a behavioral adaptation of mosquito populations to increased LLIN coverage have been observed, indicating increased selective pressure. Continued and more regular insecticide resistance monitoring (e.g., every 1-2 years) across different sites in PNG would enable the control program to detect reduced susceptibility early and adjust guidelines to prevent widespread resistance.

Background

The use of pyrethroid insecticides has dramatically increased in PNG over the last decade primarily as a result of the free nationwide distribution of deltamethrin-impregnated long-lasting insecticide treated bed nets (LLIN) [1]. Alongside case-management using rapid-diagnostic tests and artemisinin-based combination therapy, LLIN are the primary malaria control strategy implemented in PNG. Recent studies conducted by the Papua New Guinea Institute of Medical Research (PNGIMR) suggest that LLIN have significantly contributed to the declining malaria burden in the country [2,3].

Considerable insecticide resistance has arisen in *Anopheles* populations in other malaria endemic nations, particularly in Africa, following the wide-spread use of LLINs [4]. Until now, pyrethroid resistance has not been documented in PNG. In a 2010 study conducted by the PNGIMR, mosquito populations from Madang, East Sepik and Manus provinces in PNG were screened for phenotypic resistance using bioassays, and also for the knock-down resistance (*kdr*) mutation which is associated with DDT and pyrethroid resistance [5,6]. The study found all sampled mosquitoes to be fully susceptible. However, since this study was conducted over 6 years ago a reassessment of the situation is overdue, in particular when considering that the continued nationwide roll-out of LLIN has the potential to exert a strong selection pressure on local mosquito populations. Some evidence for mosquito selection attributable to LLIN has recently been published by the PNGIMR showing a shift in the peak biting times of *Anopheles farauti* from later to earlier in the evening, presumably as a consequence of people being protected by the nets at the usual peak biting hours [7]. Further analyses of related data suggested that as a consequence of this shift, the personal protection provided by LLIN decreased over time being lowest in the adult population, who may be an important reservoir for transmission [8].

As vector control is one of the cornerstones of malaria control, it is critically important to maintain insecticide resistance monitoring on a regular basis to detect a potential early build-up of resistance within vector populations, and, if detected, inform the National Malaria Control Program (NMCP) on how to revise control strategy to maintain vector control efficiency.

The present study, conducted by the Entomology Section of PNGIMR, and with the support of the Global Fund to Fight Aids, Tuberculosis and Malaria, is aimed at providing an update on the insecticide resistance situation in high malaria risk areas in PNG. The work has been conducted in the context of the independent evaluation of the Global Fund-supported NMCP by the PNGIMR [9]

The study was conducted on *Anopheles* populations in four provinces (Madang, Milne Bay, East Sepik and East New Britain) and included insecticide resistance bioassays based on WHO methodology. It thus closely resembles the last monitoring study from 2010 [6]. Three insecticides were selected for the testing i) **Deltamethrin**, which is the pyrethroid insecticide used on the LLIN distributed in PNG; ii) **Lambda-cyhalothrin**, another pyrethroid insecticide used for indoor residual spraying in some areas of the country, in particular in East New Britain and iii) Dichlorodiphenyltrichloroethane (**DDT**), an organochloride insecticide widely used in historical malaria control programs that can share cross-resistance with pyrethroids [10].

Methodology

The present study was aimed at confirming mosquito susceptibility against three common insecticides in phenotypic bioassays.

Study Locations

Four malaria endemic coastal provinces were chosen to conduct mosquito larvae collections. The reason for choosing these areas was mainly their history of insecticide use for vector control. Within each province, a number of separate locations were sampled for *Anopheles* mosquito larvae.

Figure 1 shows the sampling locations in the following four provinces of PNG:

- **Madang Province:** Amele, Transgogol and areas in and around Sausi (Ramu valley)
- **Milne Bay Province:** areas in and around Alotau
- **East Sepik Province:** areas in and around Wewak, Dreikikir, Wosera area
- **East New Britain Province:** areas in and around Kokopo

Sampling was conducted between December 2015 and June 2016 for seven days in each region. GPS coordinates were collected for each sampling location using a GPS tracker (Garmin, USA).



Figure 1: Map of PNG with study provinces shaded red. The sampling locations are indicated as red dots.

Mosquito Collection & Rearing Techniques

This study required the collection of high numbers of *Anopheles* mosquito larvae from breeding sites in the selected locations. Furthermore, a diverse range of breeding sites should be screened for mosquito larvae, in order to maximize genetic diversity and thus increase the probability to detect insecticide resistance, if present.

Potential breeding sites that can harbour *Anopheles* larvae include i) River/stream banks where flow is reduced; ii) permanent ponds with vegetation; iii) permanent wells and irrigation systems; iv) small and temporary water bodies, rainfall-related (tire tracks, puddles and drains); v) blocked stream mouths with stagnant water; vi) flooded cow paddocks and pig pens and others.

Figure 2 shows an example of the study team collecting mosquito larvae in Madang province.



Figure 2: PNGIMR entomology team collecting *Anopheles* mosquito larvae in a tyre track in Madang province (Photograph: Cyrille Czeher).

Larvae were transported to a nearby insectary if available (e.g., in Madang), or a temporary field insectary set up for rearing mosquitoes and conducting the bioassays. Larvae were placed in trays (approx. 20 x 30 x 15 cm) containing approximately 400 mL of creek water. Larvae were fed with fish food (Anderson's Foodland) until pupae developed. The pupae were collected into plastic cups using a Pasteur pipette. The cups were placed into plastic mosquito containers with a permeable net on the top. Ten (10) weight percent sugar (Ramu sugar) solution soaked cotton wool was placed on top of the net for mosquitoes to feed. The containers were covered with a moist towel to maintain adequate humidity. Mosquitoes hatched from the pupae were allowed to age 2-5 days until they were used in the bioassays. Some of the equipment described is shown in Figures 3 and 4.

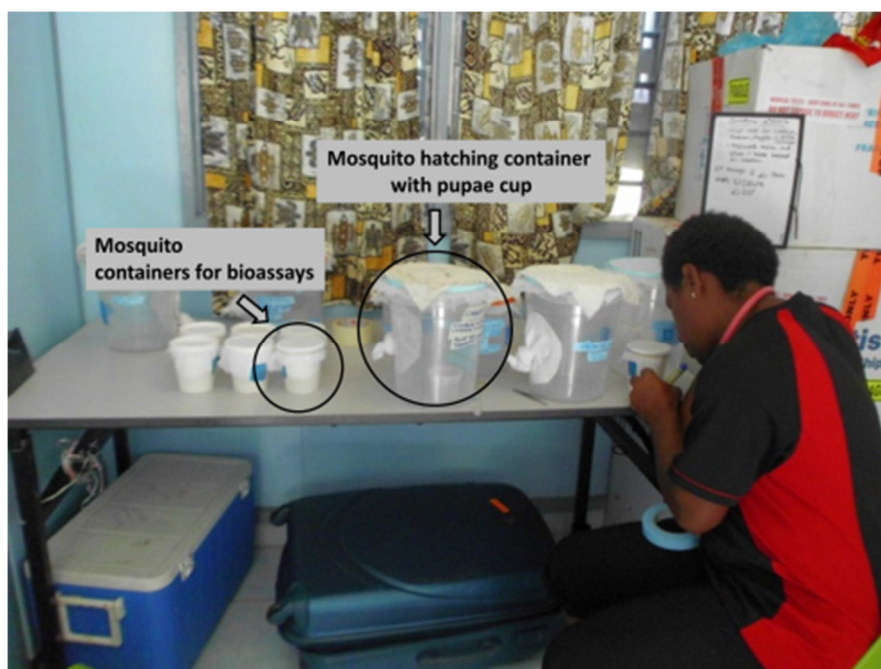


Figure 3: Larger containers were used for mosquito hatching, while smaller cups were used to transfer female mosquitoes to the bioassay tubes (Photograph: Cyrille Czeher).



Figure 4: Trays with creek water (right) were used to mature mosquito larvae (Photograph: Cyrille Czeher).

Phenotypic Bioassays

The phenotypic bioassay applied in this study has been developed by the WHO and has been previously described [11]. The WHO susceptibility test is widely used across the world, and has the advantage that it is standardized, allowing for meaningful comparisons between studies. Bioassays were conducted in standard tubes (Figure 5) using 20-30 adult female mosquitoes aged 2-5 days. To account for variability caused by e.g. mosquito age and physiological status, we aimed at conducting at least 4 replicates and 2 controls per site, meaning that between 120-180 female mosquitoes were needed per collection site.

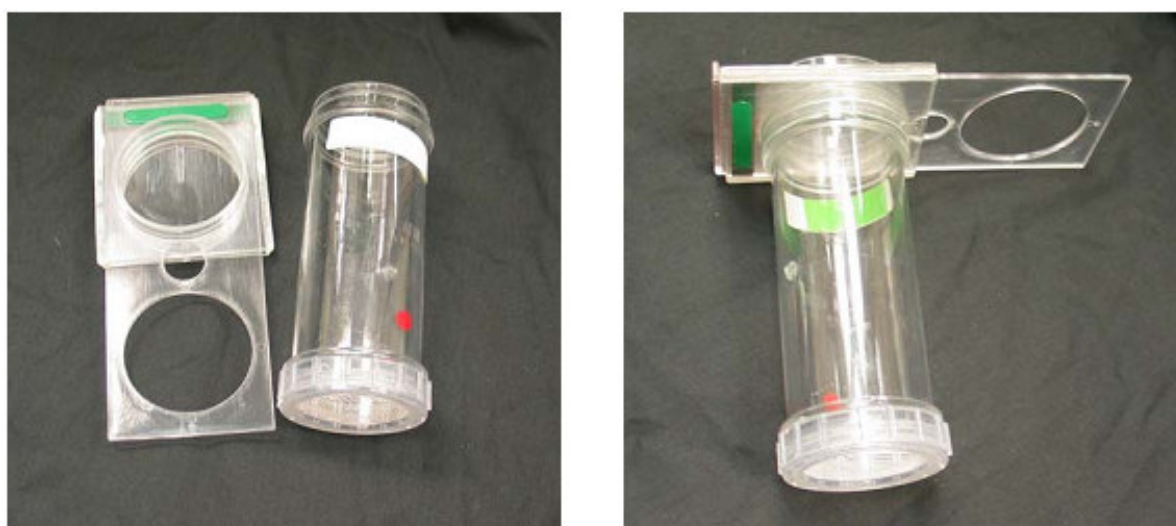


Figure 5: WHO insecticide resistance testing tube and slide unit [11].

Female mosquitoes were aspirated from the rearing containers and placed into the susceptibility test tubes containing standard papers impregnated with the discriminating insecticide concentrations conferring to a 99.9% death rate in susceptible mosquitoes over 60 minutes (0.05% Deltamethrin, 0.05% Lambda-cyhalothrin and 4% DDT, respectively) [11]. The number of knocked down mosquitoes was counted in time intervals of 5 minutes until 60 minutes were reached. The mosquitoes were transferred back to an insecticide free environment (small cup shown in Figure 3) and allowed to recover for 24h.

Molecular Mosquito Species Identification

Molecular assays were conducted to confirm morphological species identification. The results are presented in Table 1. Overall morphological species identification was good for *An. punctulatus* with nearly 80% correctly identified, acceptable for *An. farauti* with 70% correctly identified, but poor for *An. koliensis* with only 43% correctly identified. *An. koliensis* is difficult to distinguish morphologically so these results are not surprising. These results are unlikely to impact the insecticide resistance results.

Table 1: Agreement between morphological and molecular species determination.

		Molecular species analysis				
		<i>An. punctulatus</i>	<i>An. farauti</i>	<i>An. hinesorum</i>	<i>An. koliensis</i>	Total
<i>Morphology</i>	<i>An. punctulatus</i>	306 (79%)	3 (1%)	4 (1%)	76(20%)	389
	<i>An. farauti</i>	8 (4%)	149 (69%)	43 (20%)	17 (8%)	217
	<i>An. koliensis</i>	1 (1%)	22 (13%)	76 (44%)	74 (43%)	173
	Total	315	174	123	167	

Ethical Considerations

The entomology component of the National Malaria Control Program evaluation did not require ethical approval as no human subjects were involved. However, the study protocol was included in the broader protocol for the evaluation of the NMCP and approved by PNGIMR IRB (#1512) and MRAC (#15.21).

Results

Phenotypic Bioassays

To assess mosquito response to the three insecticides, three key parameters were calculated:

- i) time until 50% of mosquitoes were knocked down (survival curve),
- ii) percentage of mosquitoes knocked down after one hour of exposure, and
- iii) percentage of mosquitoes dead after the 24h recovery period.

Table 2 summarizes the findings for each province and each insecticide. Where applicable, values are given as mean (95% confidence intervals).

Overall, the times to achieve 50% mosquito knock-down were ~10 minutes for Deltamethrin, ~15 minutes for Lambda-cyhalothrin and 38 minutes for DDT (exact data given in Table 1). Consequently, for DDT, the knock down rate after 60 minutes of exposure was 87% (83-91%) whereas it was 100% for the two other tested insecticides. The death rate after 24 hours was 99% (97-100%) for DDT and 100% for the other two drugs. These results are well in line with studies from other countries (e.g. [12], an African study where DDT also showed significantly longer $T_{50\%}$ values in susceptible populations as compared to Deltamethrin and Lambda-cyhalothrin) and suggest that overall, the tested mosquito populations in the present study were phenotypically fully susceptible to the three tested insecticides.

Figure 6 shows the survival curve combined for all sites, stratified by the three drugs (DDT, Deltamethrin and Lambda-cyhalothrin). The response to DDT is significantly slower than that to Deltamethrin and Lambda-cyhalothrin

Figure 7 shows the results stratified by study site and insecticide. There were no relevant differences between study sites in the 50% knock-down times (despite the difference in knock down curves), 60 min knock-down proportion or the proportion of mosquitoes that were dead 24h after the 60 minute exposure (Table 2). This suggests that the susceptibility to the tested insecticides in PNG is still universal and there is no indication that resistance has started to develop in certain provinces but not in others.

Table 1: Summary of the phenotypic bioassay results stratified by drug and region

A) DDT			
Province	Time to 50% knock-down (minutes)[*]	Proportion dead 60 min[*]	Proportion dead 24 h[*]
Madang	40.67 (38.7-42.64)	0.84 (0.79-0.90)	0.99 (0.98-1.00)
Milne Bay	36.3 (34.89-37.71)	0.95 (0.88-1.01)	0.99 (0.97-1.00)
East New Britain	34.99 (33.13-36.86)	0.83 (0.75-0.91)	0.99 (0.95-1.00)
East Sepik	36.45 (35.43-37.48)	0.92 (0.88-0.97)	0.86 (0.70-1.00)
Average	37.10 (35.54-38.67)	0.88 (0.82-0.95)	0.96 (0.90-1.00)

B) Deltamethrin			
Province	Time to 50% knock-down (minutes)[*]	Proportion dead 60 min[*]	Proportion dead 24 h[*]
Madang	12.48 (12.02-12.94)	1.00	1.00
Milne Bay	11.07 (10.65-11.49)	1.00	1.00
East New Britain	9.19 (8.99-9.40)	1.00	1.00
East Sepik	17.72 (17.00-18.43)	1.00	1.00
Average	12.61 (12.17-13.07)	1.00	1.00

C) Lambda-cyhalothrin			
Province	Time to 50% knock-down (minutes)[*]	Proportion dead 60 min[*]	Proportion dead 24 h[*]
Madang	13.28 (12.53-14.02)	1.00	1.00
Milne Bay	17.76 (16.85-18.68)	1.00	1.00
East New Britain	14.69 (14.26-15.11)	1.00	1.00
East Sepik	18.73 (18.10-19.35)	1.00	1.00
Average	15.10 (15.4 -16.8)	1.00	1.00

^{*}given as mean (95% confidence interval)

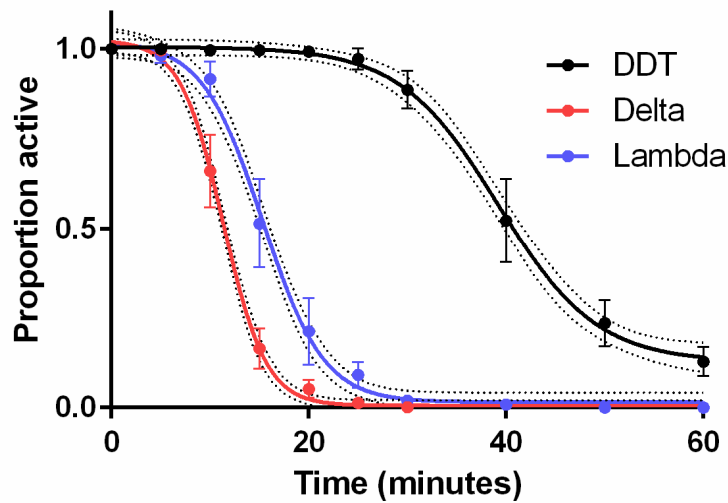


Figure 6: Proportion of active mosquitoes during 60 min of exposure to the three insecticides. Dotted lines are the 95% confidence intervals of the fit curves. Error bars denote the range of measured values

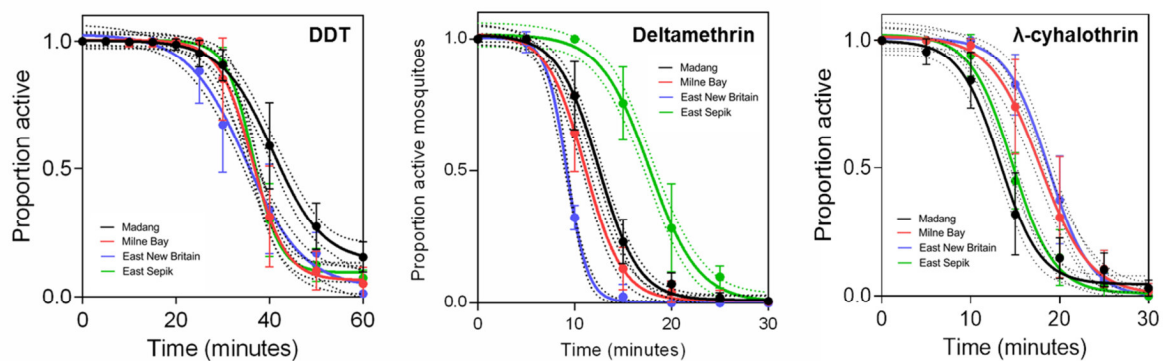


Figure 7: Proportion of active mosquitoes during 60 min of exposure to DDT (A), deltamethrin (B), lambda-cyhalothrin (C). Dotted lines are the 95% confidence intervals of the fit curves. Error bars denote the range of measured values

Discussion and Conclusions

No phenotypic insecticide resistance was identified in the present study across four provinces of PNG. The results indicate that the mosquitoes tested were fully susceptible to all three tested insecticides. While this is promising, it cannot be concluded that insecticide resistance may not have arisen in other parts of PNG or may arise in the near future, especially with the continued large-scale distribution of insecticide treated nets. Insecticide resistance is already posing a serious challenge to malaria control programs in other parts of the world. Continued regular monitoring of insecticide resistance in sentinel sites across the country is therefore warranted within the frame of routine activities of the NMCP. Full use should be made of relevant in-country capacities, including the expertise and resources available at PNGIMR and at the Malaria Surveillance and Control Unit (MSCU).

Study Challenges and Limitations

Challenges encountered in the present study included i) community participation (agreement to collect larvae was denied by community leaders in certain villages); (ii) adverse weather events (heavy rainfalls), delaying and complicating collections particularly in Madang province; and (iii) insufficient larvae yield for conducting bioassays in some sites despite extensive screening..

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