Knockdown Resistance, *rdl* Alleles, and the Annual Entomological Inoculation Rate of Wild Mosquito Populations from Lower Moshi, Northern Tanzania

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ABSTRACT

Aim: Understanding vector behavioral response due to ecological factors is important in the control of disease vectors. This study was conducted to determine the knockdown resistance (*kdr*) alleles, dieldrin resistance alleles, and entomological inoculation rates (EIRs) of malaria vectors in lower Moshi irrigation schemes for the mitigation of disease transmission. **Materials and Methods:** The study was longitudinal design conducted for 14 months. Mosquitoes were collected fortnightly by using a CDC miniature light trap in 20 houses. Mosquitoes were identified morphologically in the field, of which 10% of this population was identified to species level by using molecular techniques. Samples from this study population were taken for *kdr* and resistance to dieldrin (*rdl*) genes detection. **Results:** A total of 6220 mosquitoes were collected by using a light trap, of which 86.0% (*n*=5350) were *Anopheles gambiae sensu lato* and 14.0% (*n*=870) were *Culex quinquefasciatus.* Ten percent of the *An. gambiae s.l.* (*n*=535) collected were taken for species identification, of which 99.8% (*n*=534) were identified as *An. arabiensis* while 0.2% (*n*=1) were *An. gambiae sensu stricto.* Of the selected mosquitoes, 3.5% (*n*=19) were sporozoite positive. None of the mosquitoes tested had the *kdr* gene. The *rdl* resistant allele was detected at a frequency of 0.48 throughout the year. EIR was determined to be 0.54 ib/trap/year. **Conclusion:** The findings of this study suggest that the homozygous and the heterozygous resistance present in *rdl* genes demonstrated the effect of pesticide residues on resistance selection pressure in mosquitoes. A better insecticide usage protocol needs to be developed for farmers to use in order to avoid excessive use of pesticides.

Key words: *An. arabiensis*, EIR, Knockdown mutation, Moshi, *rdl* locus, Tanzania

INTRODUCTION

Insecticides have been the primary method of controlling disease vectors and agricultural pests for many decades. While insect resistance to pesticides has been described for decades as well, there has been a dramatic increase in resistance in recent years.[1] The relationship between resistance and insecticides has commonly been assumed to be a direct one.[1-3] In addition, the presence and persistence of chemical residues in soil and water have been detected and their impacts on aquatic fauna, including insecticide resistance of immature vector stages, have been documented.[4,5] Recent studies corroborated these assumptions by demonstrating a strong relationship between the resistance level in *Anopheles* species and the pesticide uses in crops, especially in areas with cotton and vegetable production.[6-8] In addition, metabolic resistance due to the introduction of xenobiotics in aquatic larval habitats was documented in a strain of *Aedes aegypti*.[9]

The preferential breeding sites for *Anopheles arabiensis* are rice fields and the feeding behavior of this species is largely exophilic and zoophilic.[10] In northern Tanzania, a variety of pesticides and other chemicals are sprayed by pest control services in agriculture such as organochlorides such as Dichlorodiphenyltrichloroethane (DDT) and dieldrin and in public health and veterinary pyrethroid-

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based insecticides such as permethrin and deltamethrin.[11] These are applied in rice fields once every 3 months (i.e., January, April, July, and October) following the rice irrigation cycles. These applications rarely exceed 10 times a year for other crops.[11] The presence of dieldrin, γ-HCH (lindane), and DDT residues in water and in soil have been reported in northern Tanzania,[12] more specifically in the rice-irrigated areas of lower Moshi where low levels of the three compounds were found in both water and soil.[12] In contrast, malaria vector control in this area has relied exclusively on conventional insecticide treatments and long-lasting insecticide-treated nets impregnated with pyrethroids. This strategy seems to limit the development of resistance in *Anopheles* species[13] compared with insecticide spraying.[14] Pyrethroids are also used in the region for veterinary purposes against ticks and tsetse flies in livestock and other domesticated animals.[10]

A major concern on the use of pyrethroids, especially in long lasting insecticides treated bed nets (LLITNs) and conventionally insecticides treated bed nets (ITNs),[15,16] is the lack of proper surveillance of the knockdown resistance (*kdr*) mutation that confers cross-resistance to both pyrethroids and DDT. The *kdr* mutations are known to exhibit seasonal variations throughout studies conducted in other parts of East Africa.[13,17] These variations have been attributed to several factors including interaction of vector populations with insecticide residues within the ecosystem. In order to better implement vector control strategies, resistance levels in *An. arabiensis* from areas utilizing rice irrigation schemes in the lower Moshi area have been evaluated using WHO susceptibility tests[18] to monitor the presence of *kdr* mutations[19] and to evaluate the biochemical mechanisms of resistance.[20] The previous studies showed high susceptibility in *Anopheles* populations to pyrethroids, a low frequency of *kdr* mutation (0.16%) and highly elevated oxidases and beta-esterase enzymes.[18-21] The *kdr* mutation was a substitution of a leucine by a phenylalanine in position 1014 of the sodium channel domain II segment 6 gene (L1014P). However, the most widespread *kdr* mutation in *An. gambiae sensu stricto* and *An. arabiensis* in East African populations is a substitution of a leucine by a serine in position 1014 (L1014S).[14,22] The L1014P mutation also exists at low frequencies in Kenya[13] and Uganda.[17,22]

The exposure of *An. arabiensis* to insecticides could occur at the larval stage where they come in contact with freshly sprayed or persistent molecules in the breeding sites and at the adult stage through contact with pesticides used in agriculture or during veterinary, medical, or domestic use.[2,4] Therefore, this study aimed at utilizing longitudinal monitoring of *kdr* and *rdl* alleles due to a reported reduction in *An. gambiae sensu lato* susceptibility to permethrin and other pyrethroids[18,21] and a low *kdr* frequency in a cross-sectional study in this study area.[19] Due to cyclodiene residues being found in soil and water within the lower Moshi area, the dieldrin (*rdl*) locus of the GABA receptor, the main target for cyclodiene compounds, was genotyped.

MATERIALS AND METHODS

Study area description

The study site was located in lower Moshi in the Kilimanjaro region of the northern Tanzania in an area utilizing a rice irrigation scheme (3°21'S, 37°21'E). A more detailed description of the study area can be found elsewhere.[23] Meteorological data were obtained from the Kilimanjaro International Airport Meteorological Station.

Species density variation

Mosquitoes were collected from 20 houses by using standard CDC-miniature light trap collections as described previously.[24] The collections were done fortnightly for 14 months from July 2005 to August 2006. *Anopheles gambiae s.l.* were identified morphologically by using the standard key.[25]

Sporozoite detection and entomological inoculation rate

Mosquito sporozoite detection utilized the enzyme-linked immunosorbent assay (ELISA) using the protocol of Wirtz *et al*.[26] The annual entomological inoculation rate (EIR) was calculated by using the formula published in a previous work[27] for light trap collections: 1.605 × (number of sporozoite-positive mosquitoes detected by ELISA/number of mosquitoes tested) × (number of mosquitoes collected/number of collections performed) × 365.

DNA extraction

DNA was extracted from legs of 535 individual mosquitoes by using DNA Easy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer protocol for insects. DNA was eluted in a 200 μl volume.

Mosquito identification method

The polymerase chain reaction (PCR) protocol was used to perform the amplification of DNA for species identification and other molecular use.[28] Five microliters of DNA extract were amplified in a 25 μl PCR mix containing 1× Taq buffer (Qiagen, Valencia, CA, USA), 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 ng/μl of primer UN [5-GTG TGC

CCC TTC CTC GAT GT-3'], 0.25 ng/μl of primer GA [5'-CTGGTTTGGTCGGCACGTTT-3'], 0.73 ng/μl of primer AR [5'-AAGTGTCCTTCTCCATCCTA-3'], 1 ng/μl primer QD [5'-CAGACCAAGATGGTTAGTAT-3'], 0.5ng/μl primer ME [5'-TGACCAACCCACTCCCTTGA-3'], and 0.05 U/μl HotstartTaq polymerase (Qiagen, Valencia, CA, USA). The PCR was carried out with an initial step of 10 min at 94°C to activate the DNA polymerase followed by 30 cycles, each consisting of 5 min denaturation at 94°C, 30 s annealing at 50°C, and 30 s extension at 72°C; the final cycle products were extended for 10 min at 72°C. Fragments were run through an ethidium bromide 2% agarose gel and photographed under ultraviolet light illumination.

Knockdown (*kd*) allele detection

This procedure was based on a developed PCR protocol for the detection of the *kdr* mutation (L1014S) in East African *An. gambiae* complex mosquitoes.[29] Five microliters of DNA extract were amplified in a 15 μl PCR mix containing 1× Taq buffer (Qiagen, Valencia, CA, USA), 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.3 ng/μl of primer Agd1 [5'-ATAGATTCCCCGACCATG-3'] and Agd2 [5'-AGACAAGGATGATGAACC-3'], 0.5 ng/μl of primer Agd4 [5'-CTGTAGTGATAGGAAATTTA-3'] and Agd5 [5'-TTTGCATTACTTACGACTG-3'], and 0.05 U/μl HotstartTaq polymerase (Qiagen, Valencia, CA, USA). PCR was carried out with an initial step of 10 min at 95°C to activate the DNA polymerase followed by 35 cycles, each consisting of 25 s denaturation at 94°C, 20 s annealing at 55°C, and 8 s extension at 72°C. The final cycle products were extended for 10 min at 72°C. Fragments were run through an ethidium bromide 2% agarose gel and photographed under ultraviolet light illumination. A total of 535 mosquitoes were tested.

Rdl mutation detection

This procedure was based on a developed PCR protocol.[30] Five microliters of DNA extract were amplified in a 25 μl PCR mix containing 1× Taq buffer (Qiagen, Valencia, CA, USA), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.04 ng/μl of primer RdlF [5'-AGTTTGTACGTTCGATGGGTTA-3'], RdlR [5'-CCAGCAGACTGGCAAATACC-3], AARdl [5'-GCTACACCAGCACGTGATT-3'] and RdlSS [5'-CAAGACAGTAGTTACACCTAAAGC-3'], and 0.05U/μl HotstartTaq polymerase (Qiagen, Valencia, CA, USA). PCR was carried out with an initial step of 10 min at 95°C to activate the DNA polymerase followed by 35 cycles, each consisting of 45 s denaturation at 94°C, 45 s annealing at 53°C, and 45 s extension at 72°C; the final cycle products were extended for 10 min at 72°C. Fragments were run through an ethidium bromide 2% agarose gel and photographed under ultraviolet light illumination. A total of 535 mosquitoes were tested.

Ethical consideration

The ethical clearance was given by Kilimanjaro Christian Medical College of Tumaini University. Written consent was given to all participants whose houses were involved in this study for mosquitoes sampling by using CDC miniature light traps.

RESULTS

Species identification, density variation, and entomological inoculation rate

A total of 6220 mosquitoes were collected that comprised the following species: 5350 (86.0%) *An. gambiae s.l.* and 870 (14.0%) *Culex* spp. Out of 5350 *An. gambiae s.l.* collected, 10% (n=535) was randomly sampled each month and subjected to species identification. Within that population, 99.8% (n=534) were identified by PCR to be *An. arabiensis* and 0.2% (n=1) was identified as *An. gambiae s.s*. The population of mosquitoes sampled changed from 400 mosquitoes in July 2005 to 679 mosquitoes in August 2006 [Table 1]. The study started at the end of rainy season, resulting in the low numbers of mosquitoes at the beginning of the study. The mosquito numbers subsequently increased with the start of rice-growing season and the short rains that occurred in late November 2005 and long rains that started in late February 2006. Among 535 mosquitoes identified, 19 (3.5%) of the *An. arabiensis* were found to be circumsporozoite protein positive. This resulted in an annual EIR of 0.54 ib/trap/year.

Yearly fluctuations in mutation point resistance

Mutation points were detected in 477 of the 534 *An. arabiensis*, which were successfully genotyped for *kdr* 1014S and *rdl* based on diagnostic PCR results. No samples tested positive for the knockdown resistance mutation L1014S of the sodium channel gene. However, the *rdl* locus mutation was found with a resistant allele frequency of 0.48 throughout the 14 months of study. In monthly assessments, the resistant allele frequencies dropped from 0.73 and 0.70 in July and August 2005, respectively, to 0.31 and 0.32 for the same period in 2006. During this 14-month period, heterozygote proportions (standard deviation) were 33.13% (19.9) for RR (homozygote resistant), 28.88% (18.15) for SS (homozygote susceptible), and 37.99% (10.37) for RS (heterozygote resistant). However, throughout the year, the *rdl* genotype proportions

Table 1: *An. gambiae s.l.* densities and circumsporozoite protein ELISA results throughout the study period

Months and year	Total mosquitoes specimens	*Cx quinquefasciatus*	*An. gambiae s.l.*	ELISA tested	ELISA-positive specimens
3 and 17 July 2005	459	59	400	40	1
8 and 22 August 2005	343	43	300	30	1
5 and 19 September 2005	282	71	211	21	2
3 and 17 October 2005	179	59	120	12	1
7 and 21 November 2005	271	51	220	22	0
5 and 19 December 2005	258	79	179	18	1
16 and 30 January 2006	257	68	189	19	0
6 and 20 February 2006	350	48	302	30	2
6 and 20 March 2006	522	91	431	43	1
3 and 17 April 2006	530	40	490	49	2
8 and 22 May 2006	658	39	619	62	2
5 and 19 June 2006	537	87	450	45	1
3 and 17 July 2006	822	62	760	76	3
7 and 21 August 2006	752	73	679	68	2
Total	6220	870	5350	535	19

Competing interest: Nil. ELISA: Enzyme-linked immunosorbent assay

fluctuated. The proportion of homozygote-resistant genotype (RR) decreased from 57 and 48% in July and August 2005, respectively, to 15 and 12% during the same period in 2006. In contrast, homozygote-susceptible genotypes (SS) increased from 11 and 7% in July and August 2005 to 51 and 47% the following year. Heterozygote proportions (RS) showed a stable pattern varying from 31 and 45% to 34 and 40% between July/August 2005 and July/August 2006 [Figure 1]. No direct relationships were observed between these genotype fluctuations and climatic factors or insecticide use in rice fields. Among the 19 specimens found positive for *Plasmodium falciparum* circumsporozoite protein, 7 were RR, 4 were RS, and 8 were SS. In parallel evaluations, permethrin susceptibility among wild population of the *An. arabiensis* in this study area was monitored from January 2005 to August 2006 and seemed to have seasonal variation in mosquitoes' mortality and knockdown percentage [Figure 2].

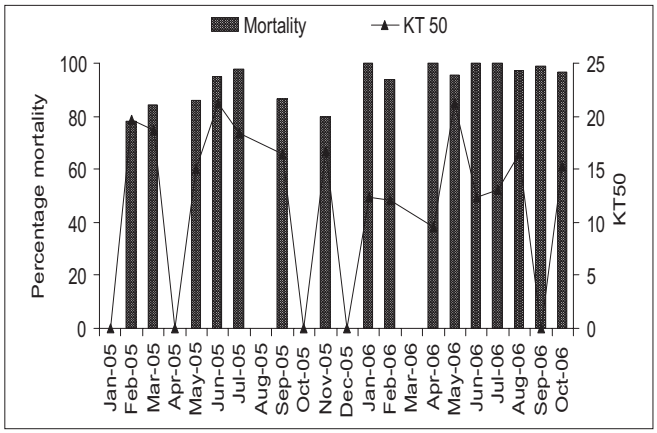

Figure 1: The proportion of genotypes at the *rdl* locus from July 2005 to August 2006 among *An. arabiensis*

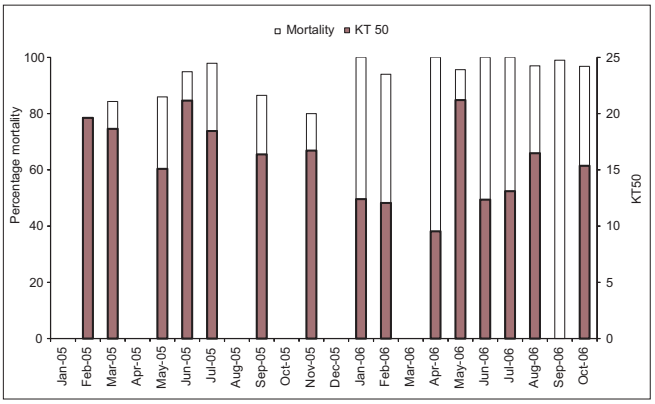

Figure 2: Variation in permethrin susceptibility among wild population of *An. arabiensis* in lower Moshi, 2005-2006

DISCUSSION

Although previous studies reported only *An. arabiensis*[31,32] in this rice-growing region of lower Moshi, Tanzania, this report demonstrates that *An. arabiensis* and *An. gambiae s.s.* are both present in this region. This is the first observation that *An. gambiae s.s.* is also present in lower Moshi. It was not observed in previous studies, but arguably it might be due to climate change and land-use practice changes.

Finding *rdl* genes in mosquitoes shows that residues of insecticides are present in the environment and likely exposed the developmental stages, confirming a previous report.[12] In addition, the absence of the L1014S *kdr* mutation was confirmed, along with low EIRs as previously reported for this region.[19,33,34] The result of this study suggests the possibility that insecticide residues in soil may have either direct or indirect impact on the development of insecticide resistance documented in other sites.[2,6]

In this same study site, metabolic resistance is known to be a major reason for reduced permethrin susceptibility among adult mosquitoes.[20] Variation of permethrin tolerance done in previous years [Figure 2] were shown to deviate within months of the years. Insecticides used for agricultural activities may have contributed to this resistance since our results show a significant increase in the mutated *rdl* allele along with yearly variations in the *An. arabiensis* population. This mutation is known to confer resistance to dieldrin, endosulfan, and lindane, all of which are used in agriculture and are found in water, sediment, and soil in the Moshi area.[12] In western Africa, a strong association was found between insecticide resistance in mosquitoes and the use of agricultural pesticides.[35] The resistant allele frequency at the *rdl* locus was found to decrease during the 14 months of the collection along with the genotype RR and an increase in genotype SS proportions. These findings suggest that the use of agricultural insecticides should be taken in consideration by public health disease vector control officials since pesticide residues in the soil contributes to the spread of resistance among disease vectors. The variations in *rdl* homozygote and heterozygote alleles have imparted a tolerance for dieldrin among mosquitoes in the rice-growing region in lower Moshi.

The Abuja declaration aims to cover 60% of the population living in malaria endemic areas of Africa with insecticide-treated bed nets by the end of 2010.[36] The absence of both West and East African *kdr* resistances in lower Moshi allows for longer performance time for pyrethroid-treated bed nets (conventionally made and/or industrially treated) and long-lasting insecticide-treated nets (LLITNs) to continue their ability to kill mosquitoes. ITNs and LLITNs show efficacy in most areas of Tanzania and other parts of Africa.[37] The active surveillance of the L1014P *kdr* mutation should be maintained in the years to come as part of the vector control program in lower Moshi and in similar agro-ecosystems. *An. arabiensis* is both zoophilic and exophilic in our study area.[10] The implementation of an active zoophylaxis is necessary as a complementary part of vector control to lower the EIR. It is therefore a viable vector control method to attract mosquitoes away from human dwellings to animals, resulting in an EIR lower than 0.54 ib/person/trap/year observed during the study.

CONCLUSION

The findings of this study suggests that agricultural, veterinary, and public health workers need to minimize pesticide residues waste in soil to prevent contaminations of the mosquito larval habitats and subsequently the evolution of resistance in malaria vectors against pesticides used for public health that have similar ingredients to those used in agriculture and veterinary services.

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