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SCIENTIFIC NOTE

THE KNOCKDOWN RESISTANCE MUTATION AND KNOCKDOWN TIME IN *ANOPHELES GAMBIAE* COLLECTED FROM MALI EVALUATED THROUGH A BOTTLE BIOASSAY AND A NOVEL INSECTICIDE-TREATED NET BIOASSAY

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ABSTRACT. Successful malaria management in Mali includes the use of pyrethroids and insecticide-treated nets (ITNs) for mosquito control; however, management is threatened by the spread of insecticide resistance detected via the knockdown resistance (*kdr*) allele. In a preliminary study, we compared the knockdown times of *Anopheles gambiae* from Mali using a novel ITN bioassay and the World Health Organization (WHO) bottle bioassay. Additionally, the frequency and relationship between *kdr* genotypes, molecular forms, and pyrethroid resistance were analyzed. The S molecular form was predominant and accounted for 76% of the assayed population. Both *kdr* resistant alleles, West Africa resistant (*kdr-w*) and East Africa resistant (*kdr-e*), were observed. There was no significant difference in knockdown time based on *kdr* genotype or molecular form of individual mosquitoes, but mosquitoes in the ITN bioassay homozygous for the *kdr-w* allele were knocked down significantly faster than those in the WHO bottle bioassay. The ITN bioassay provides an additional indicator of insecticide efficacy because ITNs, frequently used within homes, are the most common form of vector control and malaria prevention, and the ITN bioassays can evaluate seasonal field effects.

KEY WORDS *Anopheles gambiae*, insecticide resistance, Mali, knockdown resistance, insecticide-treated nets, bioassay

The notable increase in insecticide resistance, the cost of mosquito and malaria management, and the biological complexities of the *Anopheles gambiae* Giles s.s. and *Plasmodium* organisms has made both malaria and mosquito reduction a challenge. Pyrethroid insecticides combined with insecticide-treated nets (ITNs) are used to reduce both M and S molecular forms of *An. gambiae* (Favia et al. 1997) because of their strong residual properties, quick speed to knockdown, and low mammalian toxicity (Diabaté et al. 2002). Molecular detection of pyrethroid resistance occurred first in West Africa (*kdr-w*) (Martinez-Torres et al. 1998) and later in East Africa (*kdr-e*) (Ranson et al. 2000). Due to the increase in pyrethroid resistance, we conducted a preliminary study to determine if a new bioassay using ITNs was feasible, and if knockdown time and Knockdown resistance (*kdr*) genotype were correlated in either the bottle bioassay or the ITN bioassay. Our 1st objective was to compare the standard bottle bioassay design to a novel and inexpensive ITN bioassay because insecticide-treated bed-nets are the primary form of vector control in homes. Our 2nd objective was to identify the relationship of knockdown time to the frequency of the *kdr* resistant alleles in the M and S molecular forms of *An. gambiae* in the village of Pimperena, Mali.

A total of 198 female *An. gambiae* mosquitoes were collected from indoor resting sites over 5 mornings from at least 20 homes in October 2009 from Pimperena, Mali (11°28′N, 05°42′W). The collections were separated into 2 groups for 12-h bottle bioassays and 1-h knockdown trials (bottle bioassay and ITN bioassay) conducted in a temperature-controlled room (21°C) at the Malaria Research and Training Center in Bamako, Mali. For the 12-h susceptibility bottle bioassays, 3 replicates of approximately 20 mosquitoes (n = 61) were subjected to bottles coated with 0.05% deltamethrin (diluted in 1 ml of acetone) (Brogdon and McAllister 1998). Mosquitoes were held in the bottle bioassay for 3 h, and then transferred to insecticide-free cups for 12 additional hours with a constant source of sugar water. After 12 h, they were identified as dead or alive (Brogdon and McAllister 1998).

Knockdown experiments were conducted using the previously described bottle bioassay (Brogdon and McAllister 1998) or a modified ITN bioassay. The ITN bioassay used a single PermaNet 2.0-LLIN mosquito net (Vestergaard Frandsen,

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Lausanne, Switzerland), purchased from a local market within a year of being manufactured, with a targeted pretreatment dose of 1.8 g/kg of deltamethrin. The netting was lined on all sides of a 1-gallon (3.7-liter) cardboard carton, so that if a mosquito were to land, it landed only on netting. Individual bioassays were conducted with a total of 27 mosquitoes subjected individually to 1-h knockdown bottle bioassays and 110 mosquitoes subjected individually to 1-h knockdown ITN bioassays. Concurrently, known pyrethroid-susceptible *An. gambiae* (Centers for Disease Control and Prevention's G3 colony) were subjected for 3 h in the ITN susceptibility bioassay (lined with unimpregnated netting) and in bottle bioassays coated with acetone; no mortality was recorded for these controls. After the bioassays, mosquitoes were stored individually in tubes with 70% ethanol for further molecular analyses to correlate knockdown time with the observed *kdr* genotype. Mosquito DNA was extracted (Reimer et al. 2008), diagnostic polymerase chain reaction was used to identify the M/S molecular forms (Scott et al. 1993, Fanello et al. 2002), and the fragment analysis procedure (Reimer et al. 2008) was used to identify the *kdr* genotype of each mosquito. Statistical analyses were performed using SAS 9.2 software (SAS Institute Inc., Cary, NC). To compare genotype effects, descriptive statistics were used and knockdown times were log transformed and all data were analyzed with a combination of Fisher Exact Tests for direct effects and with an ANOVA (SAS Institute 2001). To determine if there was a difference in knockdown time based on molecular form, bioassay used, or *kdr* genotype, separate 1-way ANOVAs were done with the knockdown bioassay results. Only mosquitoes that were both *kdr* genotyped and identified to molecular form were used in the final analyses.

Descriptive statistics for each of the bioassays are presented in Table 1. The mean percent mortality for the 12-h bottle bioassay was 62.3% (± 6.26% SE) and a majority of the mosquitoes were homozygous for the *kdr-w* alleles (91%). Mortality comparisons of molecular forms of the 12-h bottle bioassay indicated that S-form mosquitoes had significantly more alive numbers than M-form mosquitoes (χ^2 = 6.16, df = 1, P = 0.013).

The mean percent mortality for the 1-h knockdown bottle bioassay (n = 27) was 29.6% (± 8.96% SE), and the 1-h ITN knockdown bioassay (n = 110) was 100% (± 0% SE). A majority of the mosquitoes in the 1-h bottle bioassay (74%) and 1-h ITN bioassay (64%) were S molecular form; of which these were primarily homozygous for the *kdr-w* alleles (90.1%). The ITN bioassay knockdown times were shorter than those observed in the bottle bioassay,

Table 1. Summary statistics of knockdown (KD) time (min) of *Anopheles gambiae*, comparing bottle bioassay to insecticide-treated net (ITN) bioassay.

	S molecular form				M molecular form			
	All genotypes		*kdr-w/kdr-w*		All genotypes		*kdr-w/kdr-w*	
Statistic	Bottle	ITN	Bottle	ITN	Bottle	ITN	Bottle	ITN
No. mosquitoes	23	76	20	70	4	30	1	6
$Mean_{KD}$ (± SE)	92.10 (± 11.33)	22.67 (± 0.86)	95.18 (± 12.01)	22.25 (± 0.90)	104.81 (± 15.83)	20.62 (± 1.06)	82.67	23.5 (± 3.06)
Min_{KD}	16.75	2	23.67	2	75.67	9	—	13
Max_{KD}	180	39	180	39	143.67	32	—	32
Two-tailed t-$test_{df}$ (P-value)	t_{97} = 10.92 ($P < 0.0001$)		t_{88} = 11.13 ($P < 0.0001$)		t_{32} = 14.18 ($P < 0.0001$)		—	

regardless of *kdr* genotype ($P < 0.0001$) (Table 1). The ANOVA results indicated molecular form ($F = 0.11$; df = 1, 118; $P = 0.7446$) and *kdr* genotype ($F = 0.28$; df = 4, 118; $P = 0.8926$) did not significantly affect mortality, as 96% of the M forms and 76% of the S forms died. Only the M molecular form had representatives of each *kdr* genotype, and there was no association between knockdown time and genotype ($P > 0.05$) (sample size for each molecular form and each genotype was insufficient to investigate all possible associations). Only bioassay (bottle or ITN bioassay) had a significant effect on knockdown times (Table 1).

Neither bioassay detected an effect of *kdr* genotype on knockdown time. In Pimperena only 6 homozygous susceptible individuals (*kdr-sus*/*kdr-sus*) were collected, 5 M molecular forms and 1 S molecular form (4%). To our knowledge, this is the first report of the *kdr-w* allele in the M molecular form and the first reports of the presence of the *kdr-e* allele in Mali. Our *kdr* resistant frequency data were similar to others in Pimperena (Reimer et al. 2008) and in Burkina Faso (Corbel et al. 2010). These data generate new questions regarding *kdr* and insecticide resistance and reasons why neither of these assays detected resistance. Often the *kdr* allele is genotyped and researchers assume resistance based on allelic frequency (Stump et al. 2004, Pinto et al. 2006, Reimer et al. 2008), even though heterozygous individuals are susceptible (Chandre et al. 2000). The *kdr* resistant allele does not guarantee resistance; rather, it increases the likelihood and/or opportunity for resistance as demonstrated in this study and others (Donnelly et al. 2009). In this study, resistance may have been missed due to age variation, field collecting conditions, and the small sample size. It is clear we still do not have a thorough understanding of the *kdr* allele, its role, and the potential impacts it has in conferring resistance.

The efficacy of ITNs is a concern to many, so it may be best to subject mosquitoes to a variety of bioassays to determine efficacy and resistance/susceptible rates. The ITNs are frequently used within homes and this new bioassay uses ITNs; consequently, the ITN bioassay may provide additional information on insecticide and net efficacy, net-to-net variation, and perhaps predict field resistance and susceptibility. Bottle bioassays if conducted in the standard manner are used as an early warning of insecticide resistance and can provide reliable comparison studies (e.g., time and space). Additional research comparing these 2 bioassays in the laboratory with known strains (e.g., resistant/susceptible) could lead to additional field trials. The ITN bioassays for insecticide resistance and ITN efficacy tests will be important as new and improved technologies are developed. Already, tunnel and hut tests have been conducted to compare the ability of different ITNs to reduce mosquito populations (Corbel et al. 2010). Evaluations have also been conducted to determine net efficacy after household use and washing (Lindblade et al. 2005), net comparison studies between different ITN manufacturers and models (Dabire et al. 2006), and in overall malaria prevention studies (Kilian et al. 2008). Insecticide resistance is occurring within the *An. gambiae* complex and it is critical to continue to monitor all forms of resistance, within all members of the complex, using a variety of methods to manage mosquito populations and minimize malaria transmission.

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