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Occurrence of Knockdown Resistance (kdr) Gene Mutation in Anopheles *gambiae* s.l. and Comparative Phenotypic Susceptibility to Synergized Natural Pyrethrum Formulation

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Abstract

Knockdown resistance (kdr) associated with single point mutation at the residue L1014 in the IIS6 transmembrane segment of the voltage gated sodium channel (vgsc) gene in Anopheles gambiae s.l. is one of the known mechanisms of resistance against pyrethroid insecticides. This has emerged as a real threat to the continued effective use of insecticide-treated nets (ITNs) that rely mostly on pyrethroids as the active ingredient to control malaria vectors. There is, therefore, need for continuous monitoring the occurrence of vectors and development of alternative insecticide formulations as a strategy to manage kdr resistance and to sustain the use of this important technologies in malaria vector control.

Anopheles gambiae s.l. mosquitoes were collected from Kisian, Ahero and Kipsitet, which are malaria endemic sites in western area of Kenya. The sibling species were identified using polymerase chain reaction (PCR) while genotyping for kdr mutation in the IIS4-IIS6 transmembrane segment of the vgsc was done using real time PCR (RT-PCR). Susceptibility of the wild An. gambiae s.l and pink eyed An. gambiae s.s with fixed kdr resistance genes to synergized natural pyrethrum formulation was assessed using WHO impregnated papers.

Bioassay data data were subjected to analysis of variance (ANOVA) while those with coefficient of variation (CV) of >15% were transformation into logarithms before analysis. Treatment means were compared using least significant difference (LSD, P=0.05).

All the mosquitoes obtained from Kipsitet and Ahero areas were An. Arabiensis while in Kisian, 73% were An. arabiensis and 27% were An. gambiae s.s. No kdr genes were detected in the An. arabiensis while there was 100% frequency of the L1014S kdr mutation in the An. gambiae s.s. Natural pyrethrum formulation achieved significantly (P=0.0001) higher kill than pyrethroids against An. gambiae s.s. with kdr genes. High susceptibility of the An. gambiae s.s. with kdr mutation and wild phenotypes to the synergized pyrethrum formulation provides crucial evidence for practical management of the spreading kdr and other resistance mechanisms to pyrethroids in malaria vectors.

The apparent lack of kdr resistance genes detected in An. arabiensis is proposed as a subject for further research.

Keywords: Knockdown resistance (kdr) • Anopheles gambiae s.l • Phenotypic susceptibility • Pyrethrum formulation

Introduction

Trials undertaken in many African countries have shown remarkable success of insecticide treated nets (ITNs) in reducing overall mortality and morbidity associated with malaria [1-4]. Currently, the WHO Pesticides Evaluation Scheme (WHOPES) recommends only the pyrethroids as the active ingredient (a.i) for use in insecticide formulations for treatment of bed nets [5]. This was due to their efficacy against malaria vectors and relative low mammalian toxicity [6]. However, reported incidences of resistance to pyrethroid insecticides by anopheles malaria vectors threatens to reduce the potency of this important control method [7-11]. Two key mechanisms of insecticide resistance that are common include metabolic resistance and target site insensitivity [12]. The former is attributed to amplification of genes coding for a group of enzymes (eg P450 mono oxygenases) that biochemically makes the compound less toxic to the mosquito or those such as esterases that prevent reactions detrimental to normal physiological functions [12,13]. On the other hand, target site insensitivity results from one or more mutations that make the physiological target of an insecticide less reactive to the chemical [12].

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Knockdown resistance (kdr) is one of the resistance mechanisms to pyrethroids by Anopheles mosquitoes and is mainly associated with target site insensitivity arising from single point mutation in the voltage gated sodium channel (vgsc) characterized by a leucine-phenylalanine (L 1014F) mutation in West Africa [14] and leucine-serine (L1014S) mutation in East Africa [9]. While many studies have focused on assessing the occurrence and spread of kdr resistance genes in Anopheles mosquitoes [15-18] and assessing the phenotypic resistance to the existing insecticide products [18], there has not been a corresponding effort to develop new insecticide formulations as an intervention in the fight against the resistance in order to ensure sustainability of the use of ITNs in malaria control. The solution, therefore, lies in the consideration of alternative insecticide molecules or products [19], including the use of safer insecticide formulation based on synergized natural pyrethrum, Chrysanthemum cinerariaefolium. Despite their photolabilty, natural pyrethrins, when suitably formulated with a synergist and antioxidant, increase in their stability and residual efficacy [20] and may thus have different resistance selection profile to pyrethroids. It is generally accepted that, certain formulation additives like synergists act through competitive inhibition of metabolic enzymes like the P450s or by hindering site accessibility to the compound [21]. However, the potential role of a mixture of a synergist and an antioxidant in a pyrethrum formulation on kdr resistance is not known. Besides, there is also need to continuously monitor the occurrence of kdr mutation within Anopheles malaria vector population as part of the overall vector control strategy.

The ojective of this study, therefore, was to determine the comparative susceptibilty of An. gambiae s.l. mosquitoes with kdr resistance genes and wild phenptypes to synergized natural pyrethrum insecticide formulation and

spread of kdr genes in *Anopheles gambiae* s.l from three malaria endemic areas in western Kenya, namely; Kisian, Ahero and Kipsitet.

Materials and Methods

Study area

The study was conducted in three areas i.e Kisian and Ahero in Kisumu West and Nyando Districts respectively in Kisumu county and Kipsitet in Kericho county, Kenya, all being Plasmodium falciparum malaria holoendemic areas situated within Western Kenya [10]. The first two sites (Kisian and Ahero) separated with about 20 km are located in the Lake Victoria region's climatic domain at an altitude of about 1170m above sea level (a.s.l.) and receives an annual rainfall of about 700 mm. Kipsitet, on the other hand, is located at the transition zone of the lowland lake region warm climate of the Nyanza province and the highland cool climate of the Rift Valley province situated at an altitude of 2000m a.s.l. The area receives high annual rainfall of upto 2000mm and is therefore prone to occurrence of epidemic malaria outbreaks.

Mosquito collection

Indoor-resting adult Anopheles mosquitoes were collected based on procedures earlier described [22]. In brief, mosquito collection was done in houses using spotlights and aspirators in the morning hours from 8.00am-11.00am. The mosquitoes were initially identified morphologically as an gambiae s.l. or An. funestus as previously described [23]. The mosquitoes were then put in paper cups and fed with 10% sucrose solution soaked in cotton. While in the laboratory the mosquitoes were transferred into breeding cages in order to establish larger colonies from which the emerging adults were pooled together and samples used for molecular identification of the An. gambiae sibling species, genotyped for the presence of kdr genes and bioassays conducted to determination differential susceptibility of the Anopheles mosquito species to the synergized natural pyrethrum formulation, deltamethrin and permethrin.

Molecular identification and kdr genotyping

DNA extraction: The process was based on the protocol earlier described [24]. In the procedure, a +65ºC water bath was prepared, then dry samples of 30 unfed adult mosquitoes from each study site were placed individually in sterile centrifuge tubes and crushed in 100µl of grinding buffers in a 4:1 ratio i.e homogenization buffer of 0.25M EDTA, 2.5%W /V SDS and lysis buffer consisting and 0.5M Tris Base all mixed to a pH of 9.2, respectively. The ground samples were incubated in 65oC water bath for 30 minutes in order to denature nucleases that would further degrade DNA and provide optimum temperature for activity of lysis buffer. A volume of 14 µl of potassium acetate was then added and the samples vortexed to mix and then incubated in ice for 30 minutes and the supernatant transferred. The samples were then spun for 10 minutes at 13,200 rpm and the supernatant removed and stored in sterile vials. A volume of 200 µl of cold 90% absolute ethanol was then added and samples placed at -20ºC for 20 minutes. A final spin was done for 20 minutes at 13,200rpm to pellet the DNA. The samples were then reconstituted in 100 µl of TE buffer (0.001M EDTA, 0.01M Tris-HCl at pH 8.0) to remove any RNA that co-precipitated with DNA. The DNA pellets were then allowed to dry by inverting the tubes.

Molecular identification of An. gambiae species using polymerase chain reaction (PCR): Conventional PCR following protocol modified from a previous study [25] was used to distinguish between the two sibling species of the An. gambiae complex native. The protocol had the following ingredients: 5X GoTaq PCR buffer, Primers [for An. gambiae s.l. species identification, forward universal primer (5`-GCT GCG AGT TGT AGA GAT GCG-3`), reverse An. gambiae primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3`) were used, and for An. arabiensis a reverse primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3')], $MgCl_2$, dNTPs (deoxynucleoside triphosphates), Taq DNA polymerase per reaction as below (Table 1).

The reaction was ran on Perkin Elmet Gene Amp PCR system 9600 for 30 cycles at 95ºC for 30 seconds denaturation, 64ºC at 30 seconds for annealing and 72ºC for 45 seconds for elongation. The PCR products were resolved on a 3% agarose gel and visualized by Ultraviolet trans illumination.

Table 1. The reaction mixture setup of the PCR master mix/sample.

Reagent	Concentration for 1 sample	Volume per sample
PCR Water	n/a	7.025 µl
5X PCR buffer (No $MgCl_2$)	1X	3 µl
2 mM dNTP mix	0.2 mM of each	1.51 µl
25 mM $MgCl_2$	1 mM	0.6 µl
Primer GA	1 µM	0.6 µl
Primer AR	1 µM	0.6 µl
Primer UN	1 µM	0.6 µl
Taq DNA Polymerase	0.075U	0.075 µl
Total		**14 µl**

Genotyping for kdr genes: Real-time PCR (RT-PCR) was used to determine kdr genotype at the amino acid position 1014 of the voltage gated sodium channel following a method modified from the protocol described earlier [26]. In RT-PCR dNTPS, buffer, and Taq polymerase were all included in one commercial mix (2X concentration) together with allele-specific probes that bind to the PCR product during the course of the reaction and assist in distinguishing between the alleles. Samples were genotyped using probes for wild type (5'-CTTACGACTAAATTTC-3' labeled with HEX), and L1014S allele (5'-ACGCTGAATTTC-3' labeled with FAM) and L1014F allele (5' ACGACAAAATTTC-3' labeled with FAM). RT-PCR reactions were run on a Stratagene MxPro 3000 machine using a 96-well format. Each reaction included 50 µl of 2X Taqman RT-PCR master mix, 0.2 µM kdr forward primer (5'-GCTGCGAGTTGTAGAGATGCG-3'), 0.2 µM reverse primer (5'-GCTTACTGGTTTGGTCGGATGT-3'), the wild type and L1014S probes at respective concentrations of 0.2 µM and 0.15 µM and 50ng DNA template. Each 96-well plate included positive controls for all three genotypes in triplicate along with non-template negative control. PCR conditions included initial melting step at 95oC for 25 seconds and annealing and elongation at 64ºC for 1 minute. Reaction curves (Figures 1 and 2) were visualized using the Stratagene MxPro QPCR software.

Bioassays on comparative susceptibility of the wild An. gambiae s.l and pink eyed An. gambiae s.s with fixed kdr resistance genes to synergized natural pyrethrum formulation and pyrethroid insecticides

Formulation of a synergized pyrethrum emulsified concentrate: An emulsifiable concentrate (EC) formulation containing 5% pyrethrins, weight/volume (w/v), mixed with a synergist, an antioxidant and a non-ionic emulsifier was made at the Pyrethrum Board of Kenya (PBK). The active ingredient was obtained from 25% (w/v) pyrethrins extract manufactured at the same processing factory. The synergist, and antioxidant and emulsifier were commercial grades obtained from the manufacturers, Endura Spa (Italy) and Bayer AG (Germany), respectively.

Bioassay procedure: Batches of 20 unfed An. gambiae s.l. and pink eye An. gambaie ss females, 2-4 days old were exposed to filter papers impregnated with 0.75% permethrin, 0.05% deltamethrin, 1% pyrethrins technical (unsynergized) and 1% pyrethrum-formulation (synergized). The permethrin and deltamethrin papers were obtained from WHO reference centres through KEMRI while the pyrethrins were impregnated locally in the laboratory. The susceptible An. gambiae Kisumu strain was used as the reference strain and tested simultaneously with the field population. In the tests, plastic tubes were lined with appropriate test paper and mosquitoes were transferred from holding cage to the tube with an aspirator. After transfer, the were placed horizontally on a flat surface to ensure maximum contact with the impregnated papers. Insecticide susceptibility was monitored every 5 minutes for 1 hour and mortality scored at 24 hours post-exposure. All the bioassays were conducted in five replicates in a randomized design. All specimens used for the bioassay were stored individually in numbered tubes with desiccant and

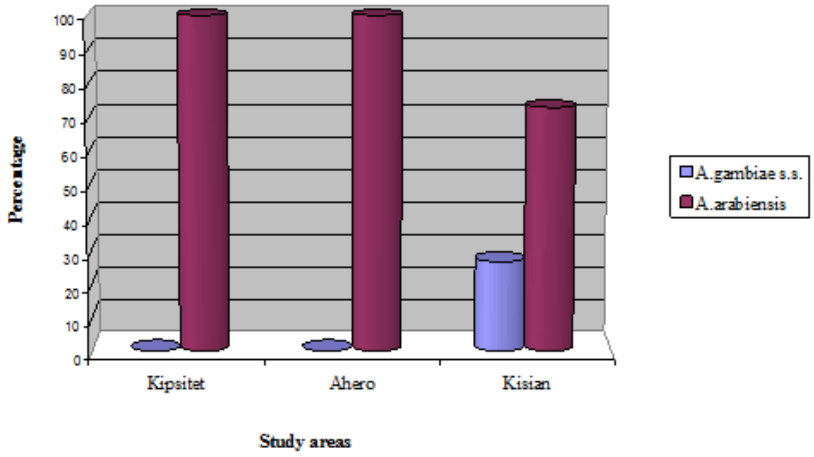

Figure 1. Comparative occurrence of a*nopheles gambiae* s.l species in Kipsitet, Ahero and Kisian areas.

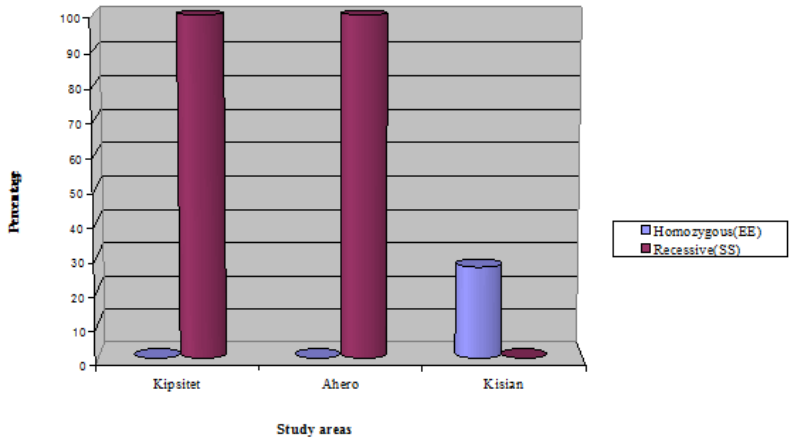

Figure 2. Spread of kdr resistance genes in Kipsitet, Ahero and Kisian areas.

preserved at 20ºC freezer for further laboratory processing. Evaluation of the resistance/susceptibility status of the mosquito vectors followed WHO [27], criteria in which resistance was indicated by mortality rates below 80%, while mortality rates greater than 98% were indicative of susceptibility. Mortality rates of between 80-98% suggested increased tolerance [27].

Data analysis: Data on counts were expressed in proportions and where the coefficient of variation (CV) was less than 15%, analysis of variance (ANOVA) of a randomized block design was conducted. While, where the CV was more than 15%, the data was transformed into logarithms to normalize the distribution and stabilize the variance before ANOVA in order to establish the effect of pyrethrum-formulation and pyrethroid insecticides on mosquitoes with kdr resistance genes and wild mosquitoes. Differences in resistance levels between the insecticides was tested using least significant difference test (LSD= ($t\alpha/2\ S\sqrt{2/n}$) at probability level of 0.05 (LSD, $P<0.05$) using SAS software [28].

Results

Determination of the *anopheles* species occurrence and kdr mutation status in Kisian, Kipsitet and Ahero areas and susceptibility of *anopheles* spp. with kdr genes to the synergized natural pyrethrum-formulation.

Species identification by PCR

PCR result on identification of the Anopheles siblings in the study areas is as shown in Figure 1. The figure shows that, 100% of the mosquito samples from Kipsitet and Ahero were An. Arabiensis while in Kisian, 73% were An. Arabiensis and 27% were An. gambiae s.s. Overall in the study areas, An. Arabiensis formed 91% of the population while An. gambiae s.s. formed only 9%.

Detection of kdr gene mutations using RT-PCR

Results on kdr gene mutation as observed from RT-PCR are presented in Figure 2. The results showed that there were no kdr genes detected in the Anopheles species that were sampled from Kipsitet and Ahero areas (Figure 2). However, in Kisian, there was 27% frequency of East African L1014S kdr mutation observed in the population and all these were detected in An. gambiae s.s. only (Figure 3 and Figure 4). The results also showed that the West african allele (L1014F) was not detected in any of the genptyped. A. gambiae individuals from the three study sites.

Effect of natural pyrethrum-formulation on pink-eyed strain anopheles gambie s.s. with fixed L1014S kdr gene mutation

ANOVA results on susceptibity of the pink-eyed An. gambiae with fixed kdr gene mutations to natural pyrethrum-formulation, unsynergised pyrethrins, permethrin and deltamethrin showed that there was significant (P=0.0001) treatment effects on knockdown and kill of the mosquitoes (Table 1). The table shows that there were significant (P=0.0001) differences in magnitudes of knockdown and kill of mosquitoes amongst the different treatments. Results further show that there was significantly (P=0.0001) lower knockdown effects of the pyrethroids (permethrin and deltamethrin) than the pyrethrum-formulation although from 15 min – 1 hour post-initial exposure, deltamethrin achieved significantly (P=0.0001) higher knockdown than permethrin. It was also observed that there was no significant (P=0.05) difference between the natural pyrthrum-formulation and the unsynergised pyrethrum in effecting knockdown of mosquitoes. Regarding mortality, Table 1 further shows that natural pyrethrum-formulation achieved significantly (P=0.0001) higher kill of 98.7% as compared to 87% with deltamethrin, and 88% with unsynergized

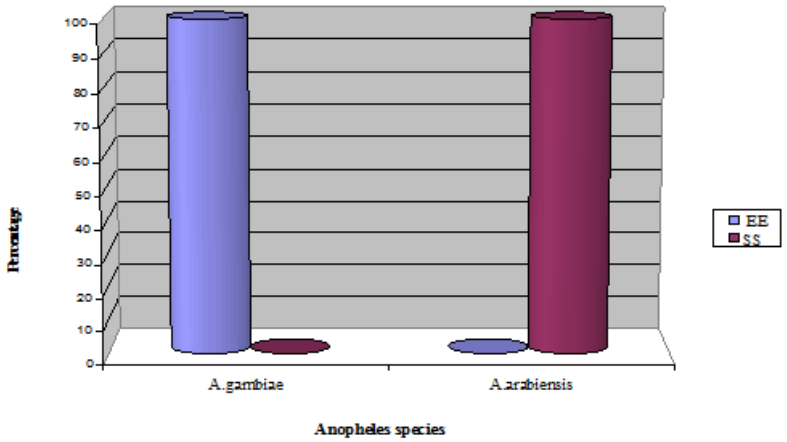

Figure 3. Occurrence of kdr resistance in *anopheles gambiae* s.l. species.

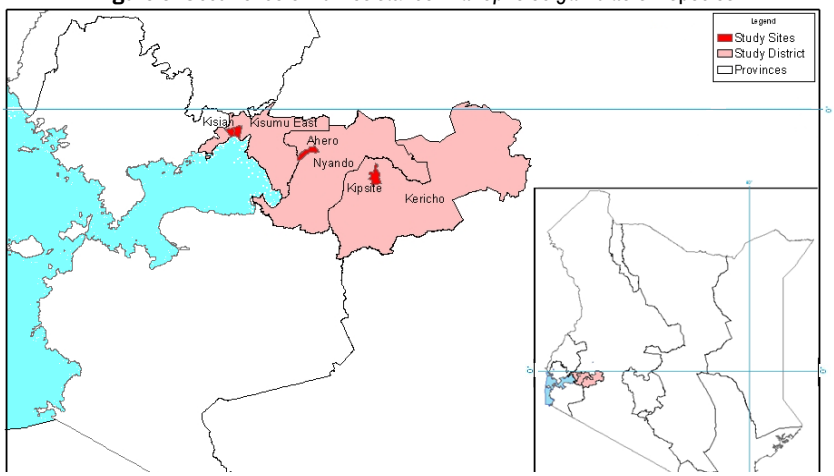

Figure 4. Appendix I. map of Kenya showing study sites for study on spread of *anopheles* species and kdr resistance mutation.

pyrethrins even though the latter two treatments were not significantly (P= 0.05) different. In all the instances, permethrin had significantly (P=0.0001) lower kill effect of only 12.5% as compared to the other treatments.

Susceptibility of wild An. gambiae s.l. from Ahero, Kipsitet and Kisian areas to natural pyrethrum-formulation, unsynergised pyrethrum, permethrin and deltamethrin.

Susceptibilty of An. gambiae ex-Ahero to natural pyrethrum-formulation and pyrethroids: Results on susceptibility of Anopheles gambiae ex-Ahero area, to the various insecticides is shown in Table 2. It was observed that the natural pyrethrum-formulation at the diagnostic dose of 1% had a very high early knockdown of 99% at 15min exposure time and this moved up to 100% within 30 min post-exposure period. At 24 hours, there was 97% mortality of the mosquitoes and this translated into a resistance index of only 3.2%, which was much below the WHO criteria of 30% mortality. The non-formulated pyrethrum 1% (technical grade) achieved a 95% early knockdown at 15 minutes, which then increased to 97% at 30 minutes. However, the product achieved only 70% mortality at 24 hours and this resulted into a resistance index of 30% proportion, which is classified as resistant as per the WHO criteria (WHO, 1998). Deltamethrin (0.5%) achieved a low early knockdown of 8% at 15 minutes exposure time but this increased to only 77% at 30 minutes and 88% after 60 minutes. The product realized 93% mortality giving only a 6.5% resistance index. Permethrin 0.75% also showed low early knockdown of 13% at 15 minutes exposure time which increased to only 57% at 30 minutes and 88% at 24 hours. The product achieved 80% mortality at 24 hours giving a 14% resistant proportion. The untreated control did not exert any mortality or knockdown.

Susceptability of An. gambiae s.s Kisumu strain (ex-KEMRI) to natural pyrethrum-formulation and pyrethroids: Results on susceptibility of laboratory bred An. gambiae s.s. ex-KEMRI to the diagnostic doses of the various insecticides are shown in Table 3. It is observed from the results that natural–pyrethrum formulation at the diagnostic dose of 1% had a very high early knockdown of 96% at 15min exposure time and this moved up to 100% from 30 minutes exposure period. At 24 hours, there was 97.5% mortality of the mosquitoes and this translated into a resistance index of 2.5%, which was much below the WHO criteria of 30% index. The non-synergized pyrethrums 1% (technical grade) achieved only a 48% early knockdown at 15 minutes which increased to 100 at 30 minutes. However, the product also achieved high mortality of 97.5% mortality at 24 hours and this resulted into a resistance index of only 2.5% which is classified as no-resistance as per the WHO criteria [27]. Deltamethrin (0.5%) achieved a low early knockdown of 29% at 15 minutes exposure time but this increased to 93% at 30 minutes and 98% after 60 minutes. The product realized 100% mortality after 24 hours. Permethrin 0.75% also showed a very low early knockdown of only 3% at 15 minutes exposure time, which increased to only 18% at 30 minutes and 65% at 24 hours. The product achieved 72.5% mortality at 27.5% resistant proportion. The untreated control did not exert any mortality or knockdown.

Susceptibility of wild An. gambiae s.l. ex-KISIAN area to natural pyrethrum-formulation and pyrethroids: Results on susceptibility of laboratory bred An. gambiae s.s. ex-Kisian to the diagnostic doses of the various insecticides are shown in Table 4. It was observed that natural pyrethrum-formulation at the diagnostic dose of 1% achieved very high knockdown levels of 100% from 15min-60minutes post treatment and 100% mortality at 24 hours, translating into a nil resistance index. The same level of response was also observed with the unformulated pyrethrins 1% (technical grade). Deltamethrin (0.5%) achieved a low early knockdown of 77.5% at 15 minutes exposure time but this increased to 95% at 30 minutes and 96% after 60 minutes. The product realized 100% mortality after 24 hours showing neither resistance nor tolerance by mosquitoes. Permethrin 0.75% on the

Table 2. Comparative susceptibility of pink eyed *an. gambiae* s.s. with kdr genes to natural pyrethrum-formulation, technical grade pyrethrum, permethrin and deltamethrin.

Treatment	%KD 3min±SE	%KD 5min±SE	%KD 10min±SE	%KD 15min±SE	%KD 30min±SE	%KD 60min±SE	%mortality±SE
Permethrin 0.75%	0.0^{b}±0.0	0.0^{b}±0.0	1.25^{bc}±1.25	1.25^{c}±1.25	6.23^{c}±2.4	6.25^{c}±3.1	12.5^{c}±1.4
Deltamethrin 0.05%	1.25^{b}±1.25	2.5^{b}±1.4	8.75^{b}±1.2	15.0^{b}±1.2	22.5^{b}±8.7	43.75^{b}±6.3	87.5^{b}±3.2
Pyrethrum- formulation 1% (synergized)	70.0^{a}±10.2	80.0^{a}±5.0	90.0^{a}±2.0	97.5^{a}±1.4	100^{a}±0.0	100^{a}±0.0	98.75^{a}±1.3
Pyrethrum extract 1% (unformulated)	82.5^{a}±6.1	83.7^{a}±2.4	91.25^{a}±2.4	96.2^{a}±2.4	98.75^{a}±1.25	96.25^{a}±1.25	88.75^{b}±3.2
Untreated	0.0^{b}±0.0	0.0^{b}±0.0	0.0^{c}±0.0	0.0^{c}±0.0	0.0^{c}±0.0	0.0^{c}±0.0	0.0^{d}±0.0
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
LSD (P α = 0.05)	14.52	9.07	8.31	8.8	12.4	9.58	7.15

Data are means (±standard error) unless otherwise stated. Month refer to the period when bioassay tests were conducted on the pyrethrins-treated nets. Data in columns represented by %KD-3 min, %KD-5 min %KD-10 min %KD- 15 min, %KD-30 mi, %KD-60 min show the proportion of mosquitoes that were knocked down after the respective minutes, following exposure to pyrethrins-treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post exposure.

Table 3. Susceptibility and resistance levels of *Anopheles gambiae* s.l. ex-Ahero area to natural pyrethrum-formulation and pyrethroids.

Insecticide	Number assayed (N)	%KD-15 min±SE	%KD-30 min±SE	%KD-60 min±SE	% Mortality±SE	% Resistant (100-%mortality)
Natural pyrethrum-formulation (1%)	100	99±1.0	100±0.0	100.±0.0	96.8±1.32	3.2
Deltamethrin 0.5%		8.0±3.0	77±6		93.15±2.3	6.5
Permethrin 0.75%	100	13.0±2.0	57±6	88.5±2.3	86.1±6.7	13.9
Unformulated pyrethrum (Technical) 1%	100	95±3.0	97±1	92.7±0.1	70±5.9	30
Untreated-control	100	0	0	0	0	

Data are means (±standard error) unless otherwise stated. Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while % KD 30 min, % KD 60 min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes respectively while % mortality refers to mosquitoes killed after 24 hours after exposure to the various insecticides. % Resistant refers to the difference between 100%kill and the realized mortality.

Table 4. Susceptibility and resistance of *anopheles gambiae* s.s. ex-KEMRI to natural pyrethrum-formulation and pyrethroids.

Insecticide	Number assayed (N)	Insecticide		Number assayed (N)	Insecticide	Number assayed (N)
Natural pyrethrum-formulation (1%)	100	96±2.0	100±0.0	98.75.±1.1	98.5±1.25	2.5
Deltamethrin 0.5%	100	29±5	93±6	98.75±1.25	100±00	0
Permethrin 0.75%	100	3±1.0	18±3.0	65±5.6	72.5±2.5	27.5
Unsynergized pyrethrum (Technical) 1%	100	48±25	100±0.0	100±0.0	97.5±0.25	2.5
Untreated-control	100	0	0	0	0	0

Data are means (±standard error) unless otherwise stated. Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while % KD 30 min, % KD 60 min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes.

Table 5. Susceptibility and resistance of *anopheles gambiae* s.l. ex-KISIAN area to pyrethrins and pyrethroids.

Insecticide	Number assayed (N)	%KD -15 min±SE	%KD-30 min±SE	%KD-60 min±SE	% Mortality±SE	% Resistant (100-% mortality)
Natural pyrethrum-formulation (1%)	100	100±0.0	100±0.0	100±0.0	100±0.0	0
Deltamethrin 0.5%	100	77.5±1.5	95±2.0	96.3±1.3	100±00	0
Permethrin 0.75%	100	2.0±2.0	40±8.0	88±4	93.0±3	7
Unsynergised pyrethrum (Technical) 1%	100	100±0.0	100±0.0	100±0.0	100±0.0	0
Untreated-control	100	0	0	0	0	0

Data are means (±standard error) unless otherwise stated. Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while% KD 15 min, % KD 30 min, % KD 60 min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes respectively while % mortality refers to mosquitoes killed after 24 hours after exposure.

other hand showed a very low early knockdown of only 2% at 15 minutes post-exposure time which increased to 40% at 30 minutes and 88% at 24 hours. The product achieved 93% mortality with only 7% resistance index. The untreated control did not exert any mortality or knockdown.

Susceptibility of Anopheles gambiae s.l. ex-KIPSITET area to natural pyrethrum-formulation and pyrethroids: Results on susceptibility of laboratory bred An. gambiae s.s. ex-Kipsitet to the various insecticides are shown in Table 5. The table shows that natural pyrethrum-formulation at the diagnostic dose of 1% achieved very high early knockdown levels of 100% from 15-60 minutes and 100% mortality at 24 hours post treatment giving a nil resistance index. The same level of response was also observed with the unformulated pyrethrum 1% (technical grade). Deltamethrin (0.5%) achieved

Table 6. Susceptibility and resistance of wild Anopheles gambiae s.l. ex-KIPSITET area to pyrethrins and pyrethroids.

Insecticide	Number assayed (N)	%KD -15 min±SE	%KD-30 min± SE	%KD-60 min±SE	%Mortality±SE	% Resistant (100-% mortality)
Natural pyrethrum-formulation (1%)	100	100±0.0	100±0.0	100±0.0	100±0.0	0
Deltamethrin 0.5%	100	6.255±2.4	35±3.5	100±0.0	97.5±1.4	2.5
Permethrin 0.75%	100	11.25±1.25	87.5±1.4	97.5±1.4	96.3±1.4	3.7
Pyrethrum (Technical) 1%	100	100±0.0	100±0.0	100±0.0	100±0.0	0
Untreated-control	100	0	0	0	0	0

Data are means (±standard error) unless otherwise stated. Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while % KD 15min % KD 30min, % KD 60min, shows the proportion of mosquitoes that were knocked down at 15, 30 and 60 minutes respectively while % mortality refers to mosquitoes killed after 24 hours after exposure.

a low early knockdown of only 6% at 15 minutes exposure time but this increased to 35% at 30 minutes and 100% after 60 minutes. The product realized 97.5% mortality after 24 hours showing no resistance index of only 2.5%. Permethrin 0.75% also showed a very low early knockdown of only 11% at 15 minutes post-exposure time which increased abruptly to 87% at 30 minutes and 97% at 24 hours. The product achieved 96.3% mortality, giving a resistance index of only 3.7%. However, the untreated control did not exert any mortality or knockdown (Table 6).

Discussion

The current study aimed at monitoring the spread of kdr genes in Anopheles malaria vectors in three malaria holoendemic areas in western Kenya and to determine the comparative susceptibilty of An. gambiae s.l. mosquitoes with kdr resistance genes and wild phenptypes to natural pyrethrum insecticide formulation developed for bed net tretment. Results showed high susceptibility of the An. gambiae s.l. including those with fixed kdr-allele mutation to the natural pyrethrum formulation showing its potential for use to manage kdr resistance in Anopheles mosquitoes. The study also revealed that, there were two key Anopheles sibling species i.e An. gambiae s.s. and An. arabiensis with much higher dominance of the latter in the study areas. There were no recorded kdr in An. arabiensis but there was near fixed presence of kdr gene mutation in An. gambiae s.s. Understanding variations in susceptibility of An. gambiae s.l. populations to different insecticides is key to successful implementation of ITN programmes in malaria control. In addition, development of resistance by Anopheles mosquitoes to insecticides used in nets has the potential to seriously compromise successful use of ITNs and malaria control in general [11,17]. The foregoing results observed are significant in planning malaria vector control initiatives since the two species are part of the major Afrotropical vectors that are efficient in transmission of Plasmodium parasites to human [29]. The occurence of kdr L1014S to near fixation in An gambiae s.s. observed in the study is in agreement with observations made earlier in studies conducted in other different sites [18]. The fixed occurrence of kdr in An. gambiae s.s. is of great challenge to effective use of ITN's since the species is known to exhibit anthropophilic and endophilic behaviour [30,31], which make it the primary target of ITNs and indoor residual spraying (IRS). The two key vector control technologies that have been shown to contribute significantly to malaria control efforts [31-33,18]. Evidence of pyrethriod insecticides failing to control An. gambiae s.l. population with high levels of kdr resistance genes has been documented [16]. The need therefore, to develop new insecticide formulations targeting the emerging kdr resistance genes in An. gambiae is crucial. Given that the two species An. gambiae s.s. and An. arabiensis occur in sympatry [34], there is no ruling out the possibility that, An. arabiensis in the study area will at one time also succumb to kdr mutation which might further complicate the whole vector control scenario. This is because, the two vector species exist in similar agro-ecosystems while the use of pyrethroids in agriculture has been tauted as a primary reason for the emergence for their resistance in An. gambiae s.l. in sub-Saharan Africa [2]. Indeed high kdr frequency has been observed in populations of An. arabiensis in Ethiopia [11] and Burkina Faso [15] resulting in negative effect in malaria vector control in those countries. Besides, among the An. gambiae complex, An. arabiensis is the most widely spread [32,35]. The lack of kdr resistance alleles observed in the species in the current study could be a result of its exophilic behaviour that exposes it less to the pyrethroids used indoors in ITN and IRS which are the current preferred vector control technologies in Kenya [32,33,18]. The significantly higher KD and mortality effect of natural pyrethrum formulation on the mosquitoes with high levels of kdr gene mutation suggest that the natural pyrethrum formulation may have different resistance selection pattern to the pyrethroids. This is especially exciting because the natural pyrethrum formulation may then be used to "boost" the failing pyrethroid based ITN's which have currently been rolled out to the masses and withdrawing them from the user communities would be very costly and logistically untenable. The need for "boosting" the nets may become a reality in the near future especially deducing from the observed low knockdown and mortality of mosquitoes with kdr gene mutation exposed to permethrin, while the molecule was the pioneer in ITNs usage and currently is an active ingredient in one of the most widely used brands of LLNs (www. olyset.com). Low knockdown effect with deltamethrin is equally of concern as this may suggest existence horizontal resistance among the pyrethroids which can have disastrous consequences. Programmes in malaria vector control though ITNs and IRS rely heavily on vector susceptibility to the available insecticides. Thus, high susceptibility of the An. gambiae s.l. to the natural pyrethrum formulation can increase the effectiveness of the ITNs especially when there is low net coverage of the population and this is pertinent because in most malaria control programmes, ITN coverage is often less than 50% [36], so reduction of vector population remain an important strategy.

The mechanism of action of the natural pyrethrum formulation on kdr resistance genes may not at this point be explicit, but the fact that the formulation had a significantly higher kill effect on mosquitoes than the unformulated natural pyrethrum may point to the role of the synergist and antioxidant that forms part of the formulation. Their addition may have other roles in influencing sensitivity of the site of action of the insecticides on the voltage gated sodium channel coupled with action on the activity of the mixed function oxidases that have previously been associated with pyrethroid resistance [12,21].

It was however encouraging from the results that An. arabiensis, still showed higher susceptibility to the natural pyrethrum formulation than permethrin implying that the natural pyrethrum formulation could still be applied in specific situations out doors for its control using a variety of space spraying techniques like fogging. The low knockdown levels exhibited by the pyrethroids on the An. gambiae s.l. may suggest emerging tolerance which may be enzyme mediated for An. arabiensis and kdr for An gambaie s.s.

The study has also shown the surging population of An. arabiensis in malaria prone areas which is a challenge to ITN and IRS currently being promoted given the exophilic behaviour of the vector and might call for redesigning the current malaria vector control strategy. The study has also shown that kdr resistance pattern is complex and dynamic process that varies with mosquito species and insecticides in use. There is thus need for deeper understanding of factors that govern population dynamics of the vectors, resistance, vector susceptibility to various insecticides in relation to available options for control.

Conclusion

In conclusion, the study has now provided an evidence based demonstration

of the ability of the natural pyrethrum formulation in controlling the *anopheles gambiae* s.l. species and standing out as a viable option for managing kdr resistance that is currently a threat to successful use of ITN to control the malaria menace. The product could thus be used to "boost" the treated nets that have been distributed in areas where pyrethroid resistance exist.

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