Scientific Research Publishing

Advances in Entomology, 2022, 10, 186-203
https://www.scirp.org/journal/ae
ISSN Online: 2331-2017
ISSN Print: 2331-1991

Evaluation of Efficacy of Insecticides and Long-Lasting Insecticidal Nets for Control of *Culex quinquefasciatus* Say Populations from Northern Nigeria

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How to cite this paper: Datti, J.A., Mukhtar, M.M., Usman, M.T., Abba, B.I., Kurfi, B.G. and Ibrahim, S.S. (2022) Evaluation of Efficacy of Insecticides and Long-Lasting Insecticidal Nets for Control of *Culex quinquefasciatus* Say Populations from Northern Nigeria. *Advances in Entomology*, **10**, 186-203.
https://doi.org/10.4236/ae.2022.102014

Received: April 2, 2022
Accepted: April 25, 2022
Published: April 28, 2022

Abstract

Information on *Culex* mosquitoes (vectors of filarial worm and viral encephalitis) from northern Nigeria is scanty, hindering evidence-based control. Here, two *Culex* populations (Kano and Kaduna) were characterized. *Culex quinquefasciatus* and *Culex pipiens* were found breeding in sympatry, with some hybrid individuals identified. Larval bioassays revealed high temephos resistance (LC_{50}s = 1.34 mg/mL and 3.01 mg/mL for Kano and Kaduna, respectively). Larvae were more sensitive to α-cypermethrin (LC_{50}s = 0.026 mg/mL and 0.067 mg/mL for Kano and Kaduna). WHO adult tube bioassays revealed high pyrethroid and DDT resistance, with mortalities of 44.01% ± 6.79%, 35.83% ± 12.58%, 29.69% ± 9.97% and 52.47% ± 4.34% for permethrin, deltamethrin, α-cypermethrin and DDT, respectively. Highest resistance was observed with bendiocarb (mortality = 13.58% ± 3.98%). High resistance was obtained with fenitrothion and malathion (mortalities = 21% ± 4.76% and 56.47% ± 8.67%, respectively), while a full susceptibility was observed with pirimiphos-methyl. Pre-exposure to piperonylbutoxide (PBO) significantly recovered α-cypermethrin susceptibility (mortality = 82% ± 5.16%, χ^2 = 50.99, $p < 0.0001$), compared with the conventional bioassay (mortality = 32 ± 7.30). Mortalities of <20% were obtained in cone bioassays with Yorkool, DuraNet and PermaNet3.0 (side panels) nets, suggesting a loss of efficacy of conventional long-lasting insecticidal nets. However, mortalities of 99% and 86% were obtained in Kano and Kaduna populations using the roof of Per-

maNet3.0 (containing PBO and deltamethrin). Despite the high frequency of the 1014F VGSC knockdown resistance mutation allele (0.90), no correlation was observed between the 1014F *kdr* genotype and resistance phenotype. Sequencing of fragments of the a*cetylcholinesterase*-1 gene detected no G119S mutation, in malathion-alive and malathion-dead females. These suggest a preeminent role of metabolic resistance in these *Culex* populations.

Keywords

Culex quinquefasciatus, Metabolic, Resistance, Insecticides, LLINs, PBO, P450s

1. Introduction

Culex quinquefasciatus Say, a member of the *Culex pipiens* group [1] is a medically important mosquito and major pest species with a worldwide distribution [2]. The species of the *Cx. pipiens* Complex particularly *Cx. quinquefasciatus* (the Southern house mosquito) found in tropical and subtropical regions of the world [3] are widespread, and predominant in the urban environment, notably in Africa, where suitable environmental conditions created by rapid, unplanned urbanization is contributing to their proliferation [4]. The *Cx. quinquefasciatus* has emerged as the most common mosquito species in major African cities [5]. In addition to the highest biting nuisance that *Culex* species could induce in most cities where it thrive in both temporary or permanent stagnant water bodies such as drains, septic tanks, wet pit latrines, organically polluted sites, puddles [6], it is known to be a major vector that transmit zoonotic diseases which affect humans and wild and domestic animals, such as lymphatic filariasis (LF) [2], St. Louis encephalitis virus (SLEV) [7], West Nile virus (WNV) [8], Zika virus [9] and Rift Valley Fever virus (RVFV) [10]. It is an opportunistic feeder, and while host choice is regionally variable, it feeds on many species of birds, mammals [11] and occasionally on reptiles and amphibians [12]. *Culex* mosquito species are known to be highly opportunistic feeding on humans and animals, a behaviour which increases their potential to transmit zoonotic diseases and makes them important threat to public health [13]. The LF caused by the parasite *Wuchereria bancrofti* is largely prevalent in sub-Saharan Africa and is one of the leading causes of long-term disability in the World [14]. As reported by Okorie and colleagues in 2013, Nigeria has a high burden of lymphatic filariasis (LF) caused by the parasite *W. bancrofti* [15]. Nigeria was reported in 2016 to have the third highest national burden of LF with estimated 114 million individual at risk of the infection. LF is among the neglected tropical diseases targeted for elimination by the World Health Organization by 2020, using mass drug administration (MDA) [16].

Available literature on the disease from both the North and Central parts of Nigeria and the report of a postal survey by the Nigerian Lymphatic Filariasis

Elimination Programme (NLFEP) have shown that lymphatic filariasis is endemic [17]. Lack of information on the distribution and degree of risk of the disease in the country are the greatest challenges confronting the NLFEP. In Africa, 34 countries are LF endemic, and Nigeria is believed to bear the highest burden, with an estimated 80 to 120 million people at risk [18]. Map studies conducted by Obiora and colleagues in 2018 demonstrated heterogeneous distribution of LF risk across the country, with northern Nigeria having more suitable environmental condition for LF occurrence [19]. Vector control is a major component of the World Health Organisation (WHO) global mosquito-borne diseases intervention strategy [20] and focuses primarily on the use of Insecticide Treated Nets (ITNs) [21], Indoor Residual Spraying (IRS) [22] and the Long-Lasting Insecticides Treated bed Nets (LLINs) [22], which are increasingly deployed in Africa, as a means of malaria control, and have the added benefit of protecting people from filarial and arboviral diseases transmitted by *culicine* mosquitoes [23]. Other control strategies involve the elimination of breeding sites and the control of mosquito larvae with larvicides which are chemical insecticides applied in the breeding sites and are the best strategy to kill larvae and pupae of mosquitoes in the water [24]. However, successful implementation of these control strategies requires prior knowledge of vector distributions, biology and changing trends on susceptibility status of the vectors.

The susceptibility of status of *Cx. quinquefasciatus* against deltamethrin insecticides has to a large extent been evaluated in the south-western part of Nigeria, for example [25], and evaluation of efficacy of deltamethrin-treated LLINs and deltamethrin-PBO treated LLINs [26] [27]. Despite the health importance of *Culex* mosquitoes it remains understudied in north-western part of Nigeria [28], with little documented evidence on its susceptibility status to guide the procurement of LLINs. Hence this study was conducted to provide baseline data on the insecticide susceptibility status of *Cx. quinquefasciatus* populations from two sites in northern Nigeria, as well as their susceptibility to the available LLINs. Synergist bioassays were carried out to investigate the role of metabolic resistance in the pyrethroid resistance. Genotyping of target site insensitivity mutations was also conducted to investigate the presence and frequency of the G119S *ace-1* mutation associated with organophosphate/carbamate resistance and the L1014F knockdown resistance (*kdr*) mutation in pyrethroid resistance.

2. Materials and Methods

2.1. Mosquito Sampling and Rearing

Larvae of *Culex* mosquitoes were collected from breeding site gutters in 2020, using classical dipping method [29]. Collections were done in two sites: 1) Dukawuya neighbourhood, (11˚58'55.60"N, 8˚29'53"E), Gwale local government area of Kano state; and 2) Tudun Wada neighbourhood, (10˚51'2"N, 7˚41'1"E), Kaduna metropolis, Kaduna state. These sites were chosen for two reasons: 1) the urban local government areas of Kano have the highest lymphatic filarial

worm prevalence in northern Nigeria [30]; and 2) comparison of Kano (Sahel savannah) with Kaduna (northern Guinea savannah) will allow capturing spatial composition of the *Culex* species, in two sites from northern Nigeria with different eco-climatic conditions, as well comparing their insecticide resistance profiles.

Strainers were used to sieve and pool together the larvae at different stages of development for bioassays. The larvae were identified as belonging to *Culex* genera using morphological keys of Gillies and Coetzee [31]. The larvae were maintained under standard insectary condition (25˚C - 28˚C and ~70% - 80% humidity, with a 12 h day/night cycle) [32] and supplied with Tetramin baby fish food. A subset of larvae from Dukawuya collection (Kano) which were used for adult bioassays was fed with 10% sucrose and randomly mixed for subsequent experiments.

2.2. Molecular Identification to Species Level

To establish the species identity of the mosquitoes, 16 adult females alive after exposure to *α*-cypermethrin and 16 dead were randomly selected and DNA-extracted [33] individually for species identification. The primers described previously [34] were utilized for PCR to identify the sibling species of the *Culex pipiens* Complex. The 15 µL reaction mix comprise 1 µL of genomic DNA, 1.5 µL of 10x TaqA Buffer, ~0.4 µM each of forward and reverse primers, 1.25 mM, of $MgCl_2$, 0.25 mM of dNTP mixes and 0.12 µL of Taq DNA polymerase, in ddH_2O. Amplification was carried out using the following conditions: initial denaturation of 5 min at 95˚C, followed by 35 cycles each of 30 s at 94˚C (denaturation), 30 s at 57˚C (primer annealing) and 1 min at 72˚C (extension). This was followed with 10 min final extension at 72˚C. The PCR amplicons were separated in a 1.5% agarose gel stained with pEqGREEN and visualized for bands.

2.3. Larval Bioassays

To profile larval resistance bioassays were conducted with six different doses (0.00001 mg/mL, 0.0001, 0.001, 0.01, 0.1 and 1.0 mg/mL) each of temephos (a well-known organophosphate larvicide) and *α*-cypermethrin (a type II pyrethroid). For each concentration, 4 replicates of 20 - 25 larvae were utilised, using the WHO procedure [35], with mortalities scored at 24 h and 48 h post-exposure. For control, 4 replicates of 20 - 25 unexposed larvae were used. Larvae were supplemented with Tetramin baby fish food.

2.4. WHO Insecticide Susceptibility Bioassay

Adult susceptibility assay was performed according to WHO guidelines [36], using a minimum of 4 replicates of 25 females (2 - 4 d old), from Kano. The mosquitoes were exposed to different public health insecticides in WHO tubes for 1 h. These include 0.75% permethrin, 0.05% deltamethrin, 0.05% *α*-cypermethrin, 4% DDT, 0.25% pirimiphos-methyl, 1% fenitrothion, 5% malathion, and 0.1% bendiocarb. For the pyrethroids and DDT, knockdown was recorded at 5 min,

10-, 15-, 30- and 60 min during exposure. Mosquitoes were transferred to holding tubes and supplied with 10% sucrose. Control mosquitoes were kept in un-impregnated papers. Mortalities were recorded at 24 h post-exposure and percentage mortalities calculate.

Synergist assay was also performed to predict the class of detoxification enzymes involved in pyrethroids resistance. Adult females were pre-exposed to PBO (4%) impregnated papers for 1 h and then immediately exposed to 0.05% *α*-cypermethrin for 1 h. For control, conventional bioassay was repeated with *α*-cypermethrin alone.

2.5. Test of Bed Nets Bioefficacy Using Cone Bioassays

A cross-sectional survey was carried out, targeting a community in Dukawuya neighbourhood, in Kano. Upon receiving informed consents, a questionnaire was administered to household respondents. The consent forms clearly explained the purpose of the research, its advantages, and the right for participants to take part in it. The respondents provide details of name (responsible adult in the house), type of bed net in the house, documentation (if available), duration/age of use of bed net, number of washes, as well as personal experience from using the net. Number of holes in the nets were also counted. Five deltamethrin-treated, Yorkool LLIN (Tianji Yorkool International, Trading Company, Ltd) and two *α*-cypermethrin-treated DuraNet (Shobikaa Impex, Private Limited) were obtained from the houses and used for the bioassays. Side panels and roof of brand new PermaNet3.0 (Vestergaard) were used as a standard control, to allow comparing the susceptibility of the *Culex* mosquitoes between used bed nets and this brand new net, frequently used in studies of bioefficacy of LLINs.

The WHO cone bioassay protocol for adult mosquitoes [37] was used to determine the bio-efficacy of the above nets on 2 - 4 d old, non-blood fed adult female *Culex* mosquitoes. Three pieces each (30 cm × 30 cm) were cut from each net, wrapped in aluminium foils and kept at 4˚C before the tests. These fragments from the above seven LLINs were used for the cone tests, using Randomized Block Experimental Design. Also, 3 fragments each from the side panels (deltamethrin only) and roof (PBO + deltamethrin) of PermaNet3.0 were also used as standard. Three cones were fixed with a plastic sheet on each of the fragments. 5 - 7 females *Culex* females were introduced into each cone placed on the LLIN and exposed for 3 min. The mosquitoes were immediately removed from the cones using a mouth aspirator, transferred into paper cups, and provided 10% sugar solution. A negative control (untreated net) was included in each series of cone tests. The mosquitoes were held for 24 h inside the cups, before mortalities were recorded.

2.6. Investigation of the 1014F kdr Mutation

Sixteen (16) adult females alive and sixteen (16) dead from *α*-cypermethrin exposure, were DNA extracted [33] and used for genotyping of 1014F knockdown resis-

tance (*kdr*) mutation, following the protocol of Martinez-Torres [38], with primers, Cdg1 (5'-GTGGAAC TTCACCGACTTC-3'), Cdg2 (5'-GCAAGGCTAAGA AAAGGTTAAG-3'), Cdg3 (5'-C CACCGTAGTGATAGGAAATTTA-3') and Cdg4 (5'-CCACCGTAGTGATAGGAAA TTTT-3'). The primary reaction contains 2.5 µL each of the primers, 1.5 µL of 10x Taq Buffer A, 0.75 µL $MgCl_2$, 0.12 µL dNTP mixes, 1 µL gDNA and 10.49 µL ddH_20. Amplification was carried out using the following conditions: initial denaturation of 1 min at 94˚C, followed by 40 cycles each of 1 min at 94˚C (denaturation), 2 min at 48˚C (primer annealing) and 2 min at 72˚C (extension). The PCR amplicons were separated in a 2% agarose gel stained with pEqGREEN and visualized under UV-light for bands.

2.7. Investigation of the G119S ace-1 Mutation

Presence of the G119S *acetylcholinesterase*-1 (*ace*-1) mutation was investigated by amplification and sequencing of fragments of the *ace*-1 gene using primers described by Weill *et al.* [39]. DNA was extracted [33] from 12 malathion-alive and -11 dead female mosquitoes and used in PCR amplification with the primers, Moustdir1 (5'-CCGGGNGCSACYATGTGGAA-3') and Moustrev1 (5'-ACGATMACGTTCTCY TCCGA-3'). The PCR was carried out in 15 µL reaction mix comprise 1 µL of genomic DNA, 1.5 µL of 10x Taq A Buffer, 0.4 µM each of forward and reverse primers, 1.25 mM, of $MgCl_2$, 0.25 mM of dNTP mixes and 0.12 µL of Taq DNA polymerase, in ddH_2O. The cycling conditions were initial denaturation at 95˚C for 3 min, followed by 35 cycles each of 94˚C for 30 s, 53˚C for 30 s and 72˚C for 1 min; and a final extension of 5 min at 72˚C. The amplicons (3 µL) were separated on a 1.5% agarose gel stained with pEqGREEN and visualized for bands. The PCR products were cleaned with a QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced on both strands with the above primers.

DNA polymorphisms were detected by manual examination of sequence traces using BioEdit version 7.2 [40]. Genetic parameters of polymorphism including number of haplotypes (h) and its diversity (H_d), number of polymorphic sites (S) and nucleotide diversity (π) were computed using DnaSP6.12.03 [41]. Haplotypes were compared by constructing a maximum likelihood phylogenetic tree, using MEGA X [42].

2.8. Data Analysis

Mortalities were corrected using the Abbott's formula [43] where more than 5% died in the control cohorts. Mortality of <80% were considered resistance (WHO criterion), >98% indicate susceptibility and between 80% - 98% suggests the possible resistance. A two-tailed Chi-Square test was conducted to establish statistical differences between synergized and synergized bioassays with α-cypermethrin. The LC_{50s} (concentrations of temephos that killed 50% of larvae) were calculated and dose-response plots created with generalised linear model (glm), using the MASS package of R

(https://rdrr.io/cran/MASS/man/dose.p.html).

3. Results

3.1. Mosquito Species Identity

To identify the sibling species of the *Culex* genus 32 females (16 alive and 16 dead from *α*-cypermethrin exposure) were PCR-identified. The top panel in Figure 1 depicts the resistant group and the bottom panel is for the susceptible. Lane 5 in top panel and lane 13 in bottom panel fails. All individuals on the top panel are *Cx. quinquefasciatus* except for individuals in lanes 6 and 15 which are hybrid of *Cx. quinquefasciatus* and *Cx. pipiens*. In the bottom panel only 3 individuals, lanes 3, 16 and 17 were of *Cx. quinquefasciatus* species, the rest were hybrid of *Cx. quinquefasciatus* and *Cx. pipiens*, except for the female in lane 4 which has additional unknown band, which is below 416 bp to be categorized as *Cx. torrentium*.

3.2. Larval Bioassays

The larval bioassays revealed resistance to *α*-cypermethrin, with mortalities of only ~25% (at 24 h and 48 h) for both populations from Kano and Kaduna (Figure 2(a) and Figure 2(b)). However, marginally higher resistance was seen with increased concentration in Kaduna population compared to Kano. The LC_{50} obtained with the Kano population (0.026 mg/mL, 95% CI: 0.019 - 0.033) is 2.6 times lower than the LC_{50} obtained from Kaduna population (0.067 mg/mL, CI: 0.058 - 0.077). For bioassays with temephos, for both populations no mortalities were observed at 24 h, and the highest mortalities obtained at 48 h were ~4% and ~9% for Kano and Kaduna populations, respectively (Figure 2(c) and Figure 2(d)). The LC_{50} for Kano population (3.01 mg/mL, CI: 0904 - 5.11), is higher, ~2.25 times than the value calculated for Kaduna population (1.34 mg/mL, CI: 0.96 - 1.72).

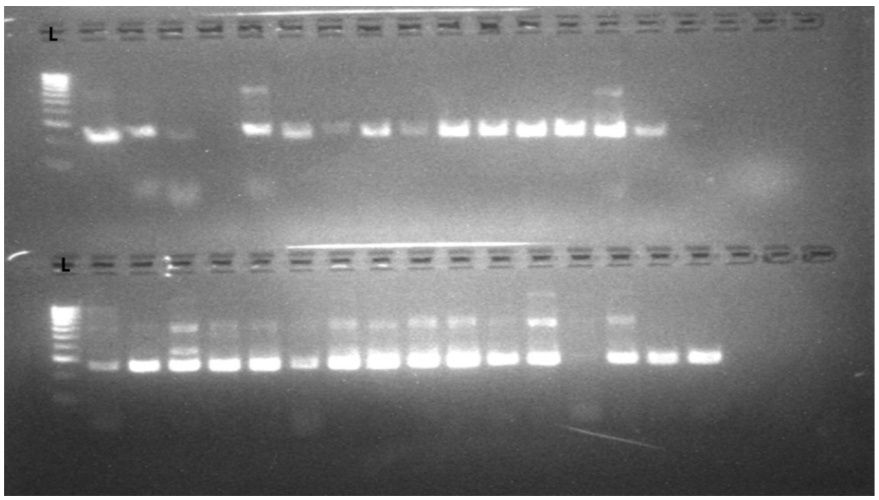

Figure 1. Agarose gel picture of PCR for species identification. Lanes 1 (L, top and bottom panels) is standard DNA ladder (hyperladder 100 bp, Bioline, 100 - 1013 bp).

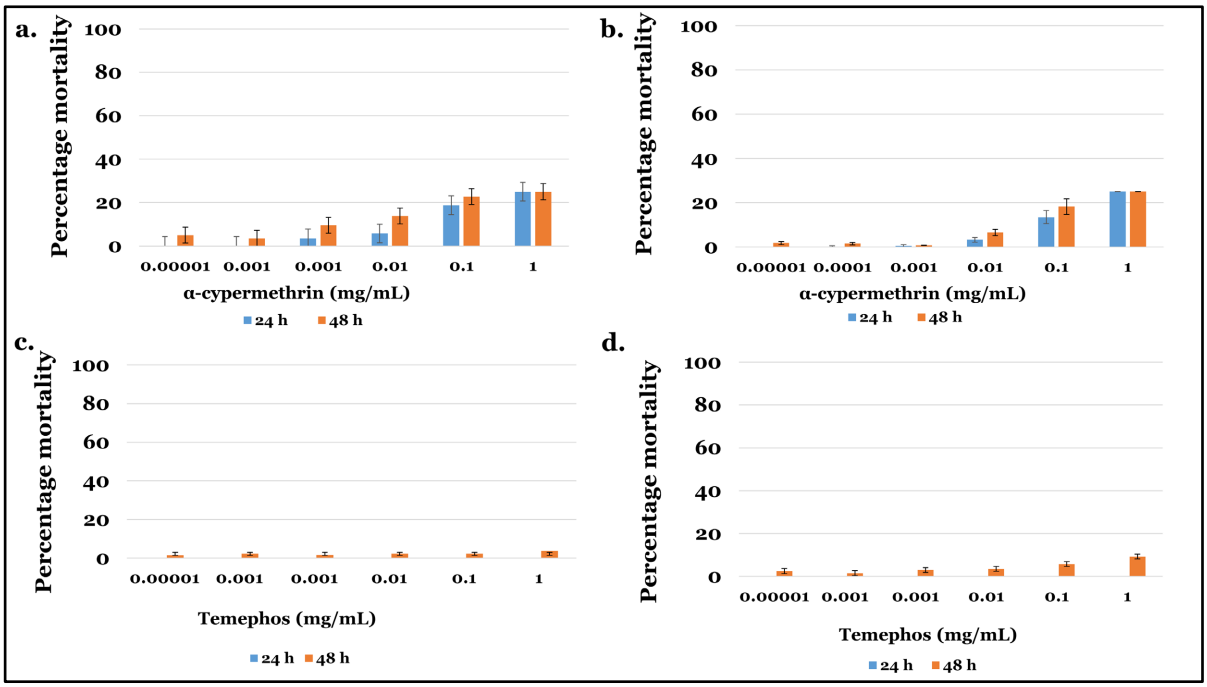

Figure 2. Results of larval bioassays with various concentrations of *α*-cypermethrin and temephos. (a) and (c) for Kano and (b) and (d) for Kaduna.

3.3. WHO Insecticide Susceptibility Bioassay

Exposure to pyrethroids and DDT reveals high insecticide tolerance, with little knockdown between 5 min and 30 min. The highest knockdown was observed with permethrin (51% at 45 min and 63% at 1 h) and *α*-cypermethrin (39% at 45 min and 41% at 1 h), while only ~20% of the females were knocked down by deltamethrin and DDT at 1 h of exposure (**Figure 3(a)**).

High resistance was observed with pyrethroids, with mortalities at 1 h of exposure of 44.01% ± 6.79% for permethrin, 35.83%± 12.58% for deltamethrin and 29.69% ± 9.97% for *α*-cypermethrin. For DDT 52.47% ± 4.34% of the female *Culex* were killed (**Figure 3(b)**). The highest resistance was observed with bendiocarb, with mortalities of 13.58% ± 3.98%. Contrasting pattern was observed with the organophosphate insecticides, with high resistance to fenitrothion (mortality = 21% ± 4.76%), high resistance to malathion (mortality = 56.47% ± 8.67%), while a full susceptibility was obtained with pirimiphos-methyl.

Prior exposure to PBO significantly recovered *α*-cypermethrin susceptibility (χ^2 = 50.99, df = 1, p < 0.0001), with mortalities in the synergized females increasing to 82% ± 5.16%, compared with the repeated conventional bioassay, which killed 32% ± 7.30% of the female mosquitoes, as **Figure 3(c)**.

3.4. Bioefficacy of Bed Nets

The LLINs utilized contained deltamethrin as active ingredient, except for DuraNet (**Table 1**). The used nets had holes except for DuraNets, and all the used nets have been washed one or more times.

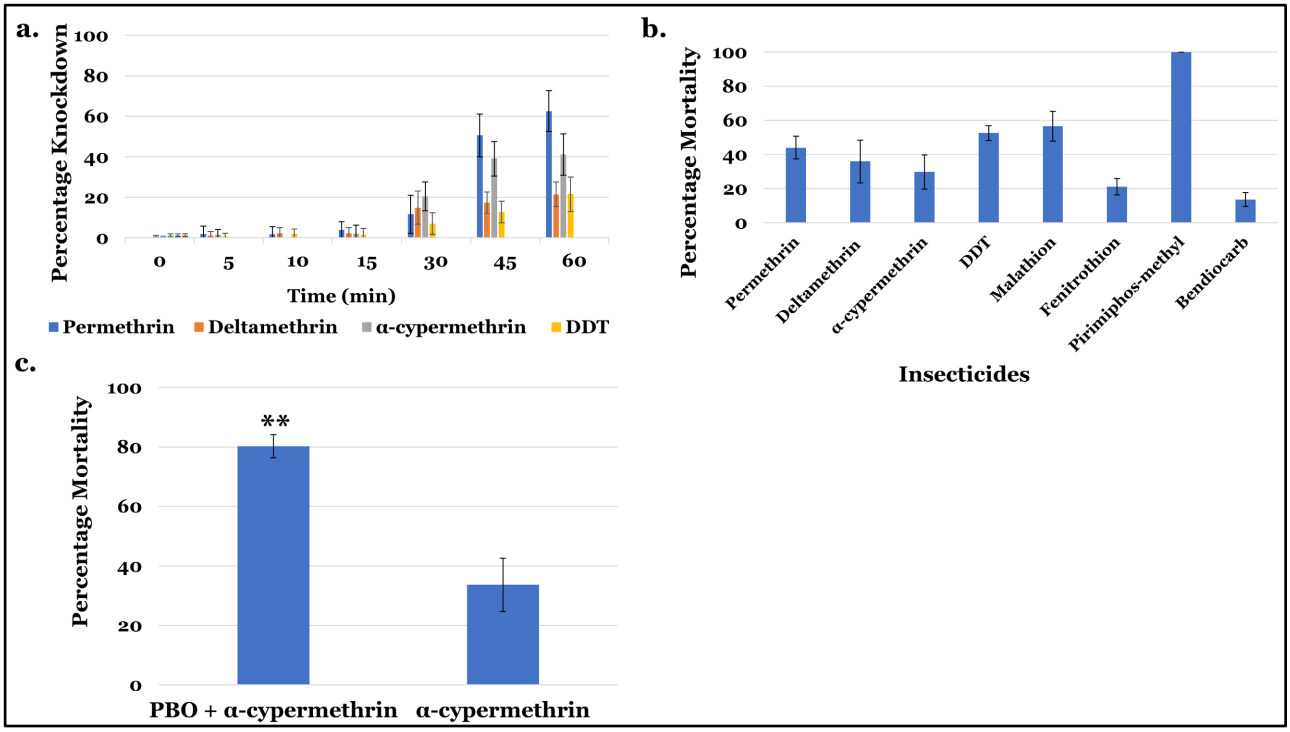

Figure 3. Results of the WHO tube bioassays with *Culex* population from Kano. (a) knockdown rate with time for pyrethroids and DDT; (b) insecticide susceptibility profiles with various insecticides; (c) effect of pre-exposure with synergist, PBO on *α*-cypermethrin susceptibility. ** = significantly different at $p < 0.0001$.

Table 1. The LLINs utilized, with the concentration of active ingredients, number of holes and washes.

LLIN brand	Insecticide	Insecticide concentration	Holes	Washes
PermaNet 3.0 side panels	Deltamethrin	55 mg/m^2 (±25%)	0	0
PermaNet 3.0 (roof)	Deltamethrin + PBO	Deltamethrin (2.8 g/kg ± 25%), PBO (4.0 g/kg ± 25%)	0	0
YORKOOL 1	Deltamethrin	55 mg/m^2	7	3
YORKOOL 2	Deltamethrin	55 mg/m^2	11	3
YORKOOL 3	Deltamethrin	55 mg/m^2	2	2
YORKOOL 4	Deltamethrin	55 mg/m^2	7	2
YORKOOL 5	Deltamethrin	55 mg/m^2	5	1
DuraNet 1	*α*-cypermethrin	55 mg/m^2	0	1
DuraNet 2	*α*-cypermethrin	55 mg/m^2	0	2

The cone bioassays revealed high resistance to the Yorkool LLINs with no mortalities for Yorkool 1, 2, 3 and 4, and only 11% and 13% for Yorkool 5, for populations from Kano and Kaduna, respectively (Figure 4).

High resistance was also observed with DuraNets, with mortalities of 16% and 14% for Kano and Kaduna populations, respectively with DuraNet 1, and ~13% each for Kano and Kaduna populations, for DuraNet 2. High resistance was also observed with the side panel of PermaNet3.0 (containing deltamethrin only), with mortalities of only 9% and 13% for Kano and Kaduna populations. This is

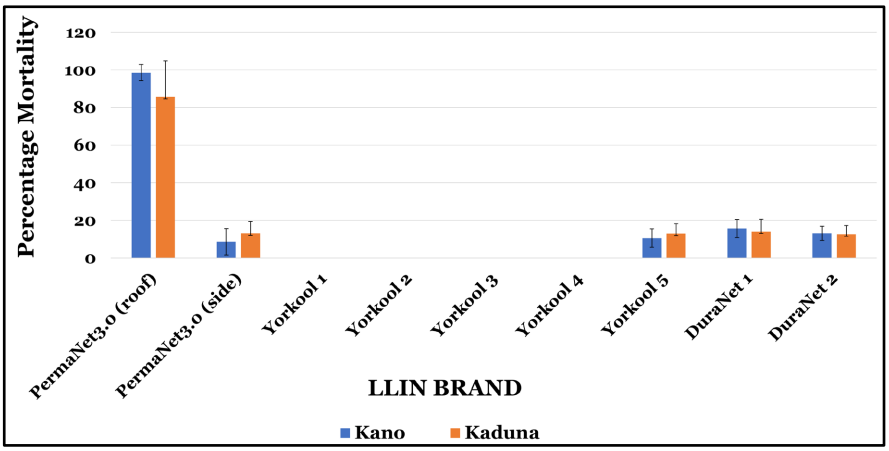

Figure 4. Bio-efficacy of various LLINs with *Culex* populations from Kano and Kaduna.

in sharp contrast to the roof of the PermaNet3.0 (containing PBO and deltamethrin) which induced mortalities of ~99% and 86% for the Kano and Kaduna populations, respectively.

3.5. Investigation of the 1014F kdr Mutation

To detect the L1014F *kdr* mutation 16 females alive from *α*-cypermethrin and 16 dead were used for genotyping using PCR. In total 15 alive females and 13 dead were successfully genotyped (**Supplementary Figure 1**). From the alive cohort 9 (60%) were homozygote resistant, RR (**Table 2**), 5 (33.33%) were heterozygotes, RS and only one female (6.66%) was homozygote susceptible (SS). From the dead, 6 individuals (46.15%) were RR, 5 were RS and 2 were SS. For both alive and the dead, 15 females were RR (53.57%) and 10 were RS (35.71%). The *kdr* genotypes were 93.33% for alive, 84.61% for dead and 89.28% for the combined alive and dead. No significant correlation was seen between the *kdr* genotype and phenotype (Odds Ratio = 2.55, $\chi^2 = 0.17$, p = 0.896).

3.6. Investigation of the G119S ace-1 Mutation

Presence of the 119S *acetylcholinesterase*-1 mutation associated with organophosphate/carbamate resistance was investigated by sequencing of fragments of *ace-1* gene from 12 malathion-resistant and 11 susceptible females. All sequences carry GGC codon specific to glycine, no of 119S replacement observed. Polymorphism analysis of the 186 bp fragment revealed a single mutation in 6 sequences from the resistant females (**Figure 5(a)**). This synonymous TAC to TAT transition specified a tyrosine residue.

The 186 bp fragment has only 2 haplotypes (the dead females having only a single haplotype, while the alive ones have 2), with a very low nucleotide diversity (0.00217) and haplotype diversity (0.403) (**Table 3**). Tajima's D was negative and not statistically significant. The major haplotype (Hap_1) comprises 17 sequences (all the sequences from dead females and 6 from alive) (**Figure 5(b)**). The sequences from Hap_1 cluster together on maximum likelihood phyloge-

netic tree, away from the 6 alive sequences with the single mutation, TAC -> TAT (Figure 5(c)).

Table 2. Phenotype and genotype frequency of the 1014F *kdr* mutation in the Kano *Culex* population.

Population	Phenotype	n	L1014F Alleles TTT (RR)	TTT/A (RS)	TTA (SS)	% *kdr* frequency (RR + RS)	*Kdr* allele	Odds Ratio (RR + RS vs SS)	χ^2 (p value)
Kano ♀	Alive	15	9 (60%)	5 (33.3%)	1 (6.6%)	14 (93.3%)	0.93	2.5 (0.2 - 3.1)	0.17 (0.89)
	Dead	13	6 (46.1%)	5 (38.4%)	2 (15.3%)	11 (84.6%)	0.84		
Total		28	15 (53.5%)	10 (35.7%)	3 (10.7%)	25 (89.2%)			

n = number of successfully genotyped individuals. Numbers in brackets represent percentage frequency. TTT: homozygote resistant alleles (RR); TTT/A: heterozygote resistant; and TTA: homozygote susceptible.

Table 3. Summary statistics for polymorphism of fragment of ace-1 from malathion-alive and dead *Culex* females.

Phenotype	N	S	h	H_d	Syn	Non-syn	π (k)	D (Tajima)	D* (Fu and Li)
Alive	12	1	2	0.54	1	0	0.0029	1.48^{ns}	0.75^{ns}
Dead	11	0	1	-	0	0	0.0000	-	-
All	23	1	2	0.40	1	0	0.0021	0.83^{ns}	0.62^{ns}

N = number of sequences (n); S, number of polymorphic sites; h, haplotype; H_d, haplotype diversity Syn, Synonymous mutations; Nonsyn, Non-synonymous mutations; π, nucleotide diversity (k = mean number of nucleotide differences); Tajima's D and Fu and Li's D statistics, ns, not significant.

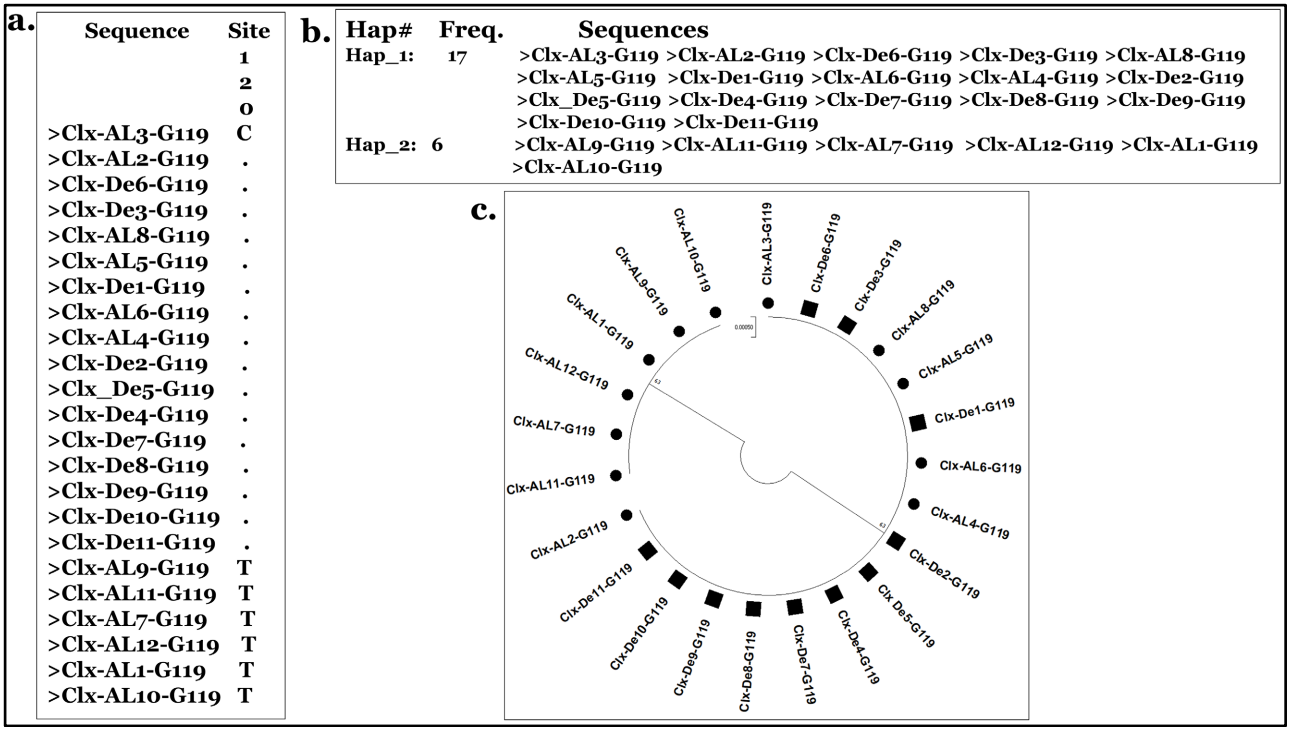

Figure 5. Polymorphism analysis of 186 bp fragment of the *acetylcholinesterase*-1 gene. (a) polymorphic sites showing C -> T transition in 6 sequences from alive females; (b) haplotypes and its frequencies; and (c) a maximum likelihood phylogenetic tree of sequences from alive and dead females.

4. Discussion

This study has investigated the susceptibility status of the *Culex* mosquitoes to different classes of insecticides and long-lasting insecticidal bed nets (LLINs) currently in use in north-western Nigeria. Large numbers of *Culex* mosquitoes were obtained in the collection during the rainy season of 2018 and 2019 in Kano and Kaduna states. *Cx. quinquefasciatus* is known to be abundant in the northern ecological zone of Nigeria [44] [45]. The result of PCR for species identification suggests a species-specific distribution of *α*-cypermethrin resistance, with the alive individuals mostly *Cx. quinquefasciatus*, while the susceptible ones are mostly the hybrids of *Cx. quinquefasciatus* and *Cx. pipiens*. But more samples are required to establish this pattern.

The larvae from both sites exhibited high temephos resistance, in contrast to the work of Delannay *et al.* [46] which established low temephos resistance in *Cx. quinquefasciatus* populations from French West Indies. The sensitivity towards *α*-cypermethrin was like the findings of Aney *et al.* [47] in populations from Bangladesh were larval mortalities of up to 70% was reported from 1 ppm exposure.

High DDT and *α*-cypermethrin resistance observed in the adult *Culex* population from Kano agrees with the findings of various studies from southern Nigeria. For example, populations from Abia state [48] were reported to be resistant to DDT, bendiocarb, and deltamethrin, with mortalities of 10.48%, 39.2% and 10.15%, respectively. But in contrast with the Ukpai and Ekedo above, in which the Abia population were resistant to pirimiphos-methyl (no mortality at all), the Kano population were fully susceptible (100% mortality). Another study recently conducted in Lagos state Nigeria [25] revealed high DDT and deltamethrin resistance, with mortalities of only 1% and 5% respectively, compared with 35.83% for deltamethrin and 52.47% for DDT, obtained from the Kano population. A study carried out in 4 sites/areas in the neighbouring Benin republic has also established high resistance in *Culex quinquefasciatus*, with mortalities ranging from 4% to 24% for permethrin, 24% to 48% for deltamethrin, 4% to 12% for DDT and 60% to 76% for bendiocarb [49]. While higher mortalities were obtained with the Kano population, mortalities of less than 20% were seen with bendiocarb, in contrast to the findings of the above study from Benin.

The synergist bioassay implicated cytochrome P450 monooxygenases in the *α*-cypermethrin resistance. The 82% recovery in mortality from preexposure to the PBO + *α*-cypermethrin is higher than the findings of Fagbohun [25] in which only 57% of the *Culex* from Lagos died from PBO + deltamethrin exposure. This together with the lack of correlation between the *kdr* genotype and resistance phenotype suggests the preeminent role of metabolic mechanism in the *Culex* population from Kano. With respect to the 1014F *kdr* mutation two studies carried out on the population from Lagos [25] [50] discovered no 1014F codon *kdr* mutation, which also suggested that its metabolic mechanisms driving pyrethroid and DDT resistance. The absence G119S *ace-1* mutations in the

Kano population further supported the suspicion that metabolic mechanism is responsible also for the malathion and fenitrothion resistance.

Not only in the used nets retrieved from the field, but even with the brand new PermaNet 3.0 (side panels), mortalities were very low, suggesting high resistance toward the pyrethroid containing LLINs in Kano *Culex*. This result is in line with the previous observation in which mortalities of only 16% were obtained when the *Cx. quinquefasciatus* mosquitoes (from Awka, Anambra state, Nigeria) were exposed to new deltamethrin-treated PermaNet 2.0 [27]. The low mortality from washed nets is not surprising as previous studies have shown progressive decreased in LLIN efficacy with increased number of washes [26] [27]. The recovery of mortalities observed from roof of PermaNet 3.0 (roof panels) suggested inhibition of P450s involved in the pyrethroid resistance. This agrees with previous observation of high mortalities induced by roof of PermaNet 3.0 in the experimental hut trial carried out in New Bussa, north-central Nigeria [26].

5. Conclusion

This study established multiple resistance in *Culex* populations from two sites in north-western Nigeria, with resistance seen towards public health insecticides from the four major classes (pyrethroids, DDT, carbamate, and organophosphate) in use in LLINs and IRS. Findings suggest the preeminent role of metabolic resistance mechanisms in play, supported by the low efficacy of conventional pyrethroid-based LLINs. The high mortalities seen with a PBO-containing LLIN suggest that the combination nets could be more efficacious against *Cx. quinquefasciatus* from northern Nigeria.

Acknowledgements

Equipment used for molecular activities carried out in this study were from a Wellcome Trust International Training Fellowship in Public Health and Tropical Medicine (WT201918/Z/16/Z) to Sulaiman S. Ibrahim.

Authors Contributions

JAD: Formal Analysis, Investigation, Methodology, Visualization, Roles/Writing—original draft, Writing—review & editing; **MMM:** Investigation, Methodology, Writing—review & editing; **MTU**; Investigation, Methodology, Writing—review & editing; **BIA**; Investigation, Methodology, Writing—review & editing; **BGK**: Supervision, Validation, Writing—review & editing; **SSI:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Funding acquisition, Supervision, Roles/Writing—original draft, Writing—review & editing.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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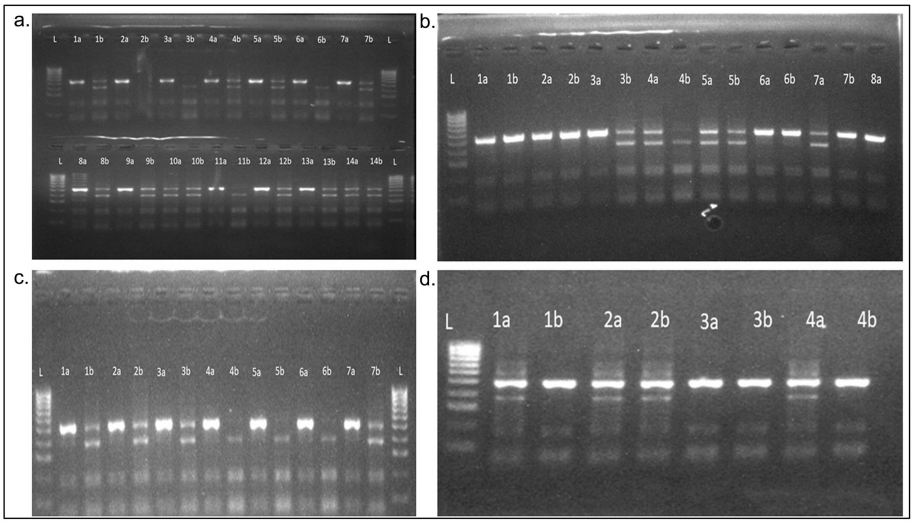

Supplementary Figure 1. The agarose gel picture of the 1014F *kdr* genotyping. a. for alive females, R, lane 2 = inconclusive, 3 = R, 4 = R, 5 = R, 6 = R, 7 = R, 8 = H, 9 = R, 10 = H, 11 = R 12 = H, 13 = R, 14 = H; b. for dead females, 1 = inconclusive, 2 = inconclusive, 3 = R, 4 = H, 5 = H, 6 = H, 7 = S; c. for dead females, 1 = H, 2 = R, 3 = R, 4 = R, 5 = R, 6 = H, 7 = R; and d. 1 = S and 2 = H both for alive, and 3 = inconclusive, while 4 = S for dead females.