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Safiyanu M
Lecturer, Department of Biochemistry, Yusuf Maitama Sule University Kano, Nigeria

Alhassan AJ
Professor, Department of Biochemistry, Bayero University Kano, Nigeria

Yayo AM
Senior Lecturer, Department of Medical Microbiology and parasitology, Bayero University Kano, Nigeria

Ibrahim SS
Senior Lecturer, department of Biochemistry Bayero University Kano, Nigeria

Imam AA
Senior Lecturer, Department of Biochemistry, Bayero University Kano, Nigeria

Abdullahi H
Lecturer I, Department of Biochemistry, Yusuf Maitama Sule University Kano, Nigeria

Correspondence
Safiyanu M
Lecturer, Department of Biochemistry, Yusuf Maitama Sule University Kano, Nigeria

Detection of KDR l1014f mutation in pyrethroids susceptible *Anopheles gambiae* S.L from Ladanai, Kano state, northwest Nigeria

Safiyanu M, Alhassan AJ, Yayo AM, Ibrahim SS, Imam AA and Abdullahi H

Abstract
Insecticides resistance has been a major challenge in malaria control effort worldwide. Understanding specie composition and their resistance mechanisms towards the major insecticides for vector control will greatly contribute in overcoming these phenomena. Larvae of anopheles mosquitoes collected from Ladanai, Nasarawa Local Govt, were reared to adults, after morphological identification of *An gambiae s.l* and their resistance profile, DNA extracts were used for determination of molecular specie composition and detection of kdr 1014F mutation. The result of the study shows that *An gambiae s.l*, the major malaria vector in the study site where highly resistant to pyretheroids permethrin and delatmethrin insecticides. Although some traces of *An gambiae ss* and *An arabiensis* were detected, *An colluzii* is the major molecular form specie identified in the site. High frequency of Kdr 1014F mutation was detected in phyretheroids susceptible *An gambiae ss* followed by *An colluzii* and least in *An arabiensis*. The finding of this study established insecticides resistance in the study site which may be associated with presence of KDR 1014F mutation triggered by indiscriminate use of insecticides for residential and agricultural purposes. Establishment of Insecticides resistance in the study site and predominance of *An colluzii* coupled with high frequency of Kdr 1014F mutation can guide the stake holders in implementing the most appropriate measures of vector control strategy in the study area.

Keywords: *An gambiae* SL, pyretheroids, 1014F mutation

1. Introduction
Malaria is a deadly disease caused by the parasite plasmodium, transmitted to human via the bites of infected female mosquitoes of anopheles genus. Sub-Saharan Africa still bears comparatively the largest malaria incidence, as the region accounts for 90% of total current malaria cases and 92% malaria deaths [1]. The Plasmodium parasites are then transfer from infected person to another through the bites of female anopheles' mosquitoes of different species (*An gambiae, An funestus, An arabiensis* etc).
Vector control intervention has yielded a significant impact in reducing the global incidence of mosquito-borne diseases [2]. This was greatly achieved in the previous years in large part due to insecticide-based strategies such as indoor residual spraying (IRS), insecticide-treated nets (ITNs) and long-lasting insecticide-treated bednets (LLITs) [2, 3]. Insecticides; pyrethroids, organochlorines, carbamates and Organo phosphates (Ops) are the only classes approved by the World Health Organization (WHO) [4, 2]. Pyrethroids and DDT share and exhibit their insecticidal effect by binding the voltage gated sodium ion channel, Carbamates and Ops on the other hand act by inactivating neuromuscular enzyme acetylcholinesterase in the nervous system [5, 6]. However, following years of intensive and indiscriminate use of insecticides in industrial, commercial and agricultural setting particularly for vector and pest control, the effectiveness of these insecticides is now threatened by the rising evolution of resistant species in target populations. Such a phenomenon is occurring throughout the whole African continent and spreads at rapid rate [7, 8].
Insecticides resistance in insects came into existence as a result of increasing occurrence of one or more resistant genes induced by exposure to various insecticides. This consequently resulted into emergence of genetic variation in the insect population created by DNA alteration, genetic recombination and gene flow. Today four major mechanisms have been

reported responsible for insecticides resistance in mosquitoes [9, 10]. (1) KDR-mediated target site insensitivity is one of the most common mechanisms of pyretheroids resistance. It occurs as result of about 11 identified VGSC mutations in mosquitoes, six of which have been functionally examined in Xenopus oocytes [6]. Pyretheroids and DDT deliver their toxic, insecticidal effect primarily by binding VGSC, altering its gating properties, and keeping it open for an unusually long time [11]. Alteration in VGSC structure, which may result to either point mutation or substitutions resulting from single nucleotide polymorphisms, eventually lead to insensitivity to DDT and pyretheroids in VGSC in the nervous system via reduction in or elimination of the binding affinity of insecticides to protein [11, 12]. Evidence from molecular, toxicological and pharmacological studies have supported the involvement of point mutation (KDR mutations) in voltage gated sodium ion channel in pyretheroids/ DDT resistance in a number of medically or agriculturally important pest species [13,12]. These evidence was summarized by Rinkevich *et al* [12] who published a comprehensive review of the current understanding of the effect of Kdr mutations in insecticides resistant insects. Among these Kdr mutations, is the substitution of leucine by phenylanine (Leu to Phe) in the sixth segment of domain II (IIS6), which was first detected in pyretheroids resistant house fly *Musca domestica* and German cockroach *Blatella germanica*. (2) Metabolic resistance, where the resistant insects degrade or detoxify the insecticides before they exert their toxic effects and (3) Resistance to penetration, where the resistant insect takes up the active substance more slowly and/or in lower quantities than the normal, sensitive insects. This study aimed at determining the frequency of KDR L1014F mutation in different anopheles species susceptible to pyretheroids in the study area.

1.1 Materials and Methods

1.1.2 Sampling site

Ladanai is located in Nasarawa Local Govt. area of Kano state and lies between 11^0 58, 37" N and 8^0 33, 45" E and total area of 34 km^2. The site is characterized by massive excavation activities, less agricultural activities and periodic cattle rearing.

1.1.3 Sample collection

Mosquito larvae were collected using soup serving spoon and white plastic bowl from Ladanai area of Nassarawa local Government area of Kano state.

1.1.4 Larval rearing

The larvae were reared in a labelled plastic tray fed with tetramin fish food until developed to pupae before transferring to cage. As the pupae emerged, they were transferred into plastic cups containing water and placed in a plastic cage where the adults emerged. The temperature and relative humidity of insectary were kept at 27°C and 70% respectively.

1.1.5 Morphological identification

Some portion of adult mosquitoes that emerged from larvae collected from Ladanai were identified using morphological characters of Gilles and Coetzee [13] under x 20 Zeiss light microscope.

1.1.6 Bioassay

Mosquitoes insecticides diagnostic kit was used to establish susceptibility and resistant status using 0.05 % deltamethrin, 0.75 % permethrin impregnated paper according to WHO procedure [14]. The knock down rate was recorded at every 15 minutes for 1hour after exposure to 0.05 % deltamethrin and 0.75 % permethrin before they were transferred back to the resting tubes for 24 hours when percentage mortality was recorded. Mortality rate between 98 -100% indicate full susceptibility, 90 -97% require further investigation, less than 90% was considered resistant to the tested insecticides. The resistant and susceptible mosquitoes were morphologically identified and transferred to labelled Eppendorf tubes and stored in silica gel for subsequent molecular identification.

1.1.7 DNA extraction

DNA was isolated from individual mosquitoes using the method of Livak [15].

Individual *An gambiae* female mosquitoes were homogenized in 100 cm^3 warmed Livak grind buffer (1.6cm^3 5M Nacl, 5.48g sucrose, 1.57g Tris, 10.16cm^3 0.5M EDTA, 2.5cm^3 20% SDS) and incubated at 65^0c for 30 min. The homogenate was briefly microfuged and 14 cm^3 8M K-acetate added, and the homogenate was incubated on ice for approximately 30mins. Debris and precipitated SDS and protein were removed by 20-mins centrifugation at 4^0c in a refrigerated centrifuge and the supernatant was transferred to new 1.5cm^3 effendorf tubes. The Nucleic acid was collected from the supernatant by adding 200µl of 100% ethanol, and was mixed and spin for 15min at 4^0c.The supernatant was removed and discarded and the pellet was rinsed in approximately 100 cm^3 ice cold 70% ethanol. The pellet was allowed to dry on bench top for approximately one hour. The dried pellet was suspended in 100 cm^3 distill water and incubated at 65^0c for 10mins.

1.1.8 Molecular specie identification

The extracted DNA pellets were subjected to polymerase chain reaction (PCR) using species specific primers for Anopheles *gambiae* complexes using sine PCR method of Santolamazza *et al* [16]. The reactions were carried out in a 15 cm^3 reaction mixture which contained 10.49 cm^3 dH_2O, 0.51 cm^3 of each Sine 200 forward and reverse primer, 0.12 cm^3 of dNTP mix, 0.75 cm^3MgCl_2, 1.5 $cm^3$10TaqA buffer, 0.12 cm^3 Kappa Taq polymerase, and 1.0 cm^3 Genomic DNA. Thermocycler conditions were 94°C for 5mins followed by thirty-five cycles of 94°C for 30 sec, 54°C for 30 s and 72°C for 60sec, with a final elongation at 72°C for 10 min, and a 4°C hold. The resulting products were electrophoresed on 1.5% ethidium bromide stained agarose gels at 80 volts for 35mins. The amplified fragments were then visualized by ultraviolet transilluminator and photographed with a syngene bio-imaging system.

1.1.9 Detection of KDR mutation

KDR west (L1014f) was detected by the method of Martinez-Torres *et al*. [17] Primers Agd1 (5'-atagattccccgaccatg-3'), Agd2 (5'-agacaaggatgatgaacc-3'), Agd3 (5'-aatttgcattacttacgaca-3') and Agd4 (5'-ctgtagtgataggaaattta-3') were used. The reaction was carried out in a 12.5cm^3 mixture constituting of 7.475 cm^3 distill water, 1cm^3 of genomic DNA, 1.25 cm^3 10 × PCR buffer A, 0.15cm^3 $MgCl_2$, 0.5cm^3 of each primer, 0.5 cm^3 of dNTP's and 0.125cm^3 of kappa Taq DNA polymerase. The initial cycling condition were set as 95°C denaturation temperature for three minutes, 10 cycles of

one minute denaturation at 94°C, 30 seconds at 54°C for annealing and 30 seconds at 72°C for extension. This was followed by 30 cycles of one minute at 94°C for denaturation, 30 seconds at 47°C for annealing and 30 seconds for extension at 72°C, and a final extension at 72°C for 10 minutes. The products of PCR were checked on a 2% agarose gel and stained with ethidium bromide for visualization using syngene bio-imaging system. The genotype frequency were determined by dividing the number of individual with a given genotype by the total number of analysed mosquitoes as follows: (i) homozygous wild type genotype frequency L1014/L1014 (ii) homozygous mutant genotype frequency F1014/F1014 and (iii) heterozygous genotype frequency L1014/F1014.

2. Results

2.1 Morphological identification

A total of 108 adult *Anopheles* reared out of the larvae collected from Ladanai were morphologically identified. The result of the study shows the presence of *An gambiae s.l*, *An funestus* and *An pharoensis* in the study sites (Table 1). The result shows the predominance of *An gambiae s.l* with 100 (92.6%) followed by *An pharoensis* 6 (5.5%) and the least is *An funestus* with 2 (1.9%). The overall sex of the mosquitoes also revealed that males represent 44 (40.3%) and females represent 64 (59.3%). Among the males 40 (91%) were *An gambiae s.l* while *An funestus and pharoensis* represent 2 (4.5%) each. In females category *An gambiae* represents 60 (93.8%) while *An funestus* and *An phronesis* represents 0 (0.0%) and 4 (6.25%) respectively. (Table 1).

Table 1: Distribution of Anopheles species Adults reared from Larvae collected from Ladanai, Nasarawa Local Govt area

Anopheles specie	**No of males identified**	**No of females identified**	**No of overall males and females**
An gambiae s.l	40 (91.0%)	60 (93.8%)	100 (92.6%)
An funestus	02 (4.5%)	00 (0.0%)	02 (1.9%)
An pharoensis	02 (4.5%)	04 (6.25%)	06 (5.5%)
Total	44 (40.7%)	64 (59.3%)	108

2.2 Bioassay

The bioassay results of female *An gambiae* s.l reared from larvae collected from Ladanai showed high resistance to permethrin and deltamethrin with 12% and 4% knock down rate respectively after one hour exposure (Figure 1) and percentage mortality of 12% and 20% respectively after 24hrs post exposure period (Figure 2).

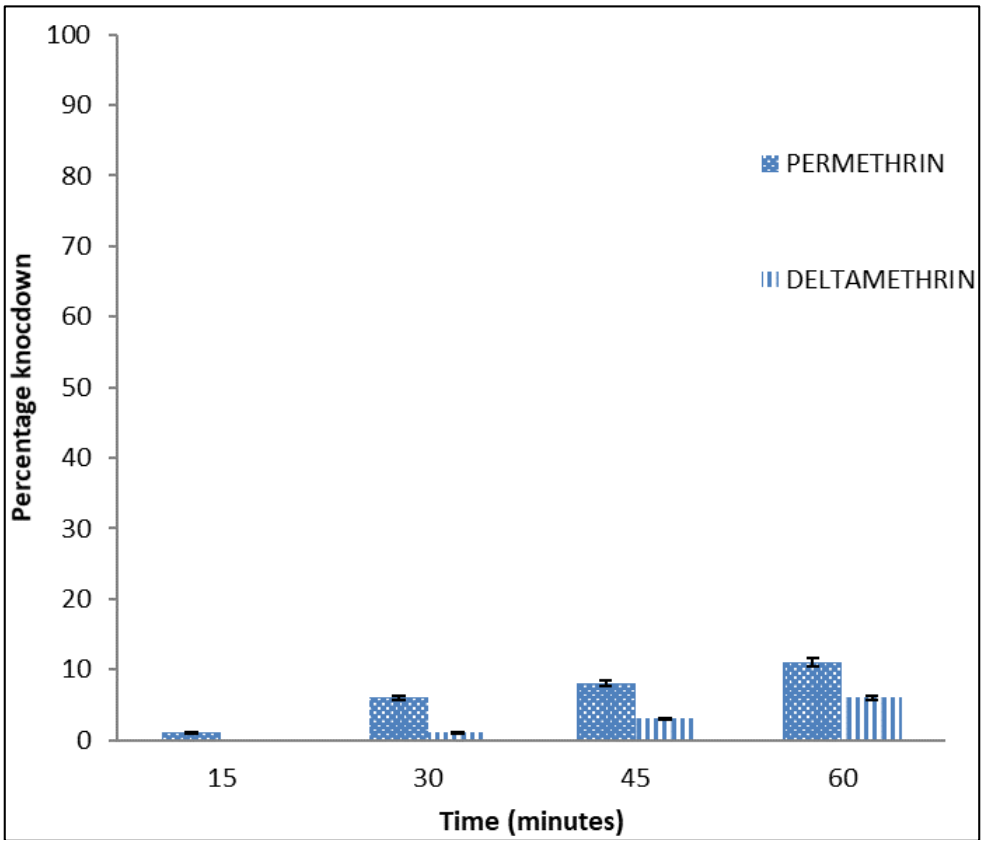

Fig 1: Knockdown profile of *An gambiae s.l* mosquitoes of Ladanai (Nasarawa LGA) Kano State. Error bars represent variabilty in data

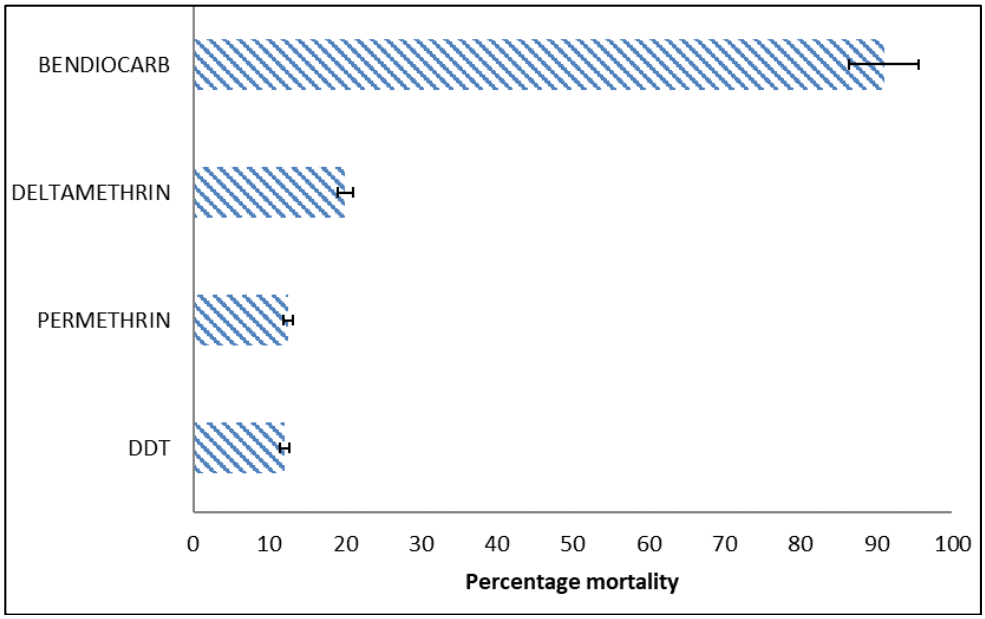

Fig 2: Insecticides suceptibility/resistance status of *Angambiae S.I* mosquitoes of Ladanai (Nasarawa LGA) Kano State

2.3 Specie composition and susceptibility status to deltamethrin

A total of 71 Female *An gambiae s.I* from Ladanai that remain alive and 19 that died after exposure to deltamethrin (figure 4) were identified using sine PCR method. The results (Table 2) show that 91% were identified as *An colluzzii*, 5.5% *An gambiae ss* and *3.3% An arabiensis*. *An colluzzii* were predominant among the mosquitoes that survived the insecticide exposure (74.4%) and *An arabiensis* were the least resistant specie (1.1%). Over all 81% (67/82) *An coluzzii*, 60% (3/5) *An gambiae ss* and 50% (1/2) *An arabiensis* were resistant to deltamethrin. On the other hand, *an arabiensis* were more susceptible to deltamethrin (50%) followed by *A gambie ss* (40%) and the least *An coluzzii* (18%).

Table 2: Molecular identification and specie composition of *An gambiae s.I* exposed to deltamethrin

Species composition	Susceptibility status	No of mosquitoes
Anopheles colluzii (91.1%)	Alive	67 (81%)
	Dead	15 (18.2%)
Anopheles gambiae ss (5.5%)	Alive	3 (60%)
	Dead	2 (40%)
Anopheles arabiensis (3.3%)	Alive	1 (50%)
	Dead	2 (50%)
Total		90

2.4 Presence KDR 1014F gene mutation in susceptible malarial vector

A total of 19 deltamethrin susceptible mosquitoes of Ladanai which include 15 *An colluzii*, 2 *An gambiae ss* and 2 *An arabiensis* were analyzed for the presence of KDR gene (Figure 3). The result shows high frequency of 1014F KDR mutant allele in *A gambiae ss* (1) followed by An *coluzzii* (0.73) and the least frequency was observed in *An arabiensis* (0.5) (Table 3). The genotype study of *An coluzzii* shows that 4 (26.6%) have homozygous wild susceptible allele 1014lL/1014L, 8 (53.3%) have heterozygous susceptible allele 1014L/1014F and 3 (20%) have homozygous resistant allele 1014F. For *An gambie ss* all the mosquitoes were heterozygous susceptible 1014L/1014F, No other genotype was detected in the sample. In *An arabiensis* 1 (50%) were homozygous susceptible 1014lL/1014L and 1 (50%) were homozygous resistant 1014F/1014F.

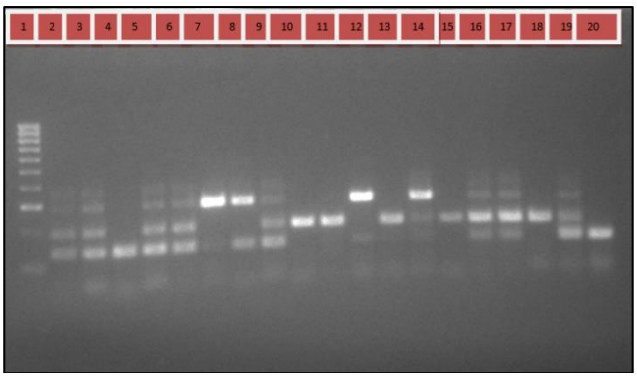

Fig 3: PCR products obtained using the AS (Allele specific) -PCR on Deltamethrin suceptible *An. gambiae s.l* after separation on a 2% agarose gel. Lane 1: 1000 bp DNA ladder Lane 4, 7,8 12and 20: homozygous wild type mosquitoes (L1014L/L1014L) (137bp); Lane 2, 3, 5,6,,9,13,14,16,17 and 19 heterozygous specimens (L1014L/ L1014F) (195/137bp); Lane 10,11,15 and 18: homozygous resistant specimen (L1014F/ L1014F) (195bp).

Table 3: Frequencies of KDR mutation in deltamethrin susceptible *An gambiae sl* of Ladanai

		Genotype			Frequency of Allele 1014F (%)
Specie	**N (%)**	**1014L/1014L n (%)**	**1014l/1014F n (%)**	**1014F/1014F n (%)**	
An colluzii	15	4 (26.6)	8 (53.3)	3 (20)	0.7
An gambiae ss	2	0.0	2 (100)	0.0	1.0
An arabiensis	2	1 (50)	0.0	1 (50)	0.5

3. Discussion

3.1 Morphological identification

The results of this study reveals the abundance of *An gambiae s.l* over other species identified in study site. This is similar to the finding of Coluzzi *et al* [18] who reported *An gambiae* as the most common and important vector of malaria parasite in sub-Saharan Africa. The results also revealed presence of other species like *An funestus and An pharoensis* in comparatively low proportion in the study sites. This supports the work of Ahmed and Ahmed [19] who reported the specie composition of Anopheles species reared from larvae collected from Nasarawa Local Govt Area as 72% *An gambiae s.l*, 29% *An funestus* And 6% *An maculipalpis*. The presence of *An funestus and An pharoensis* in relatively low proportion may be as a result of unfavorable vector survival parameters such as availability of breeding site, vegetation, climatic condition among others.

3.2 Insecticides susceptibility test

This work investigated the resistance/susceptibility status of *An gambiae s.l* specie towards pyretheroids permethrin and deltamethrin insecticides. (Figure 1 and 2). The high resistance detected in this study is similar to what was previously reported in North central and South western Nigeria [20]. The result also agree well with the report of Safiyanu *et al*., [21] who published high and moderate resistance with respect to deltamethrin and bendiocarb respectively in *An gambiae sI* collected from both residential and agricultural areas of Bichi, Kano, Northwest Nigeria. The pyretheroids resistance observed in permethrin may not be unconnected to long term use of the insecticides for indoor residual spray (IRS) and long lasting insecticides treated bed nets (LLINS) as well as their increasing application for agricultural purposes.

3.3 Specie composition and susceptibility to deltamethrin

The result of the specie composition revealed the predominance of *An coluzzii* (91.1%) followed by *An gambie ss* (3.3%) and *An arabiesis* (1.1%). This support the previous observation that *An gambiae ss* could be predominant in sudan savannah ecological zone, compared to *An arabiensis* that was spread across Sudan, sahel and northern guinea savannah [22, 23]. The result is also supported by the finding of Ibrahim *et al.* [24] who reported the predominance of *An colluzzii* (77%) over *An arabiensis* (22%) but no *An gambiae ss* was detected from Auyo town, Northwest Nigeria. The susceptibility result to deltamethrin exposure shows that 81% *An coluzzii*, 60% *An gambiae* ss and 50% *An arabiensis* were resistant to deltamethrin. Ibrahim *et al* [24] further supported the predominance of *An colluzzi* resistant to pyretheroids with 92.9 % (105/113) and 45.4% (15/33) were resistant to lambda cyhalothrin. However the result absolutely contradict the work of Yahaya *et al* [25] who published predominance of *An arabiensis* (76.8%) followed by *An merus* (22.1%) and *An gambiae ss* (1.2%) from Tanzania. This may be as a result of differences in environmental factors such as geographical location, climatic condition, pollution and socioeconomic standard.

3.4 Presence of KDR 1014F mutation in susceptible mosquitoes

The L1014F KDR mutation affects the sensitivity of sodium ion channel protein to DDT and phyretheroids insecticides. The L1014F mutation has been reported to associate with insecticide resistant in at least 39 insect species including 6 mosquitoes. Ibrahim *et al* [26] have implicated high frequency of *Kdr* 1014F with pyretheroids resistance in *Anopheles coluzzii* in Sudan savannah of northern Nigeria. Unexpectedly the result of this study revealed the presence of *Kdr* 1014F in 20% and L1014F in 53% of *An colluzzii* susceptible to deltamethrin. This suggests that the insecticides resistance may become more severe in the subsequent generation in the study site as the 1014F kdr allele is spreading and detected even in the susceptible individuals. The presence of 1014F kdr allele is supported by the recent work of Merieme *et al.* [26] who reported the presence of kdr susceptible allele 1014L in lambda cyhalotrin resistant *cx pipiens*. The presence of kdr mutation gives no indication of the actual strength of resistance level. Even if the kdr mutant gene is detected or not, the combination of other resistance mechanism like metabolic based mechanism could also play crucial role in the impact of resistance. Elevated activities of detoxification enzymes have been implicated by Safiyanu *et al.* [27] in resistance to major insecticides in *An gambiae* collected in Auyo irrigation and residential sites, Jigawa, Nigeria.

4. Conclusion

The finding of this work indicates *An gambiae s.l* as the major malarial vector in both study sites and *An coluzii* as the major molecular form in Ladanai. Resistance of mosquitoes to the major insecticides may be as a result of rapid spread of kdr 1014F mutation.

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