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Determination of Mechanism of Resistance to Pyrethroid by *Anopheles gambiae sensu lato* from Gombe State, Nigeria

Asama'u Yusuf[1], Lazarus Joseph Goje[2], Simon Gabriel Mafulul[3], Nabiha Garba[4], Nura Abubakar[5]

[1,2,4,5]Gombe State University, Nigeria; [3]University of Jos, Nigeria

lacz1980@gmail.com; jlgoje@gsu.edu.ng

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Abstract

The emergence of insecticide resistance in Anopheles gambiae sensu lato poses a significant challenge to malaria control efforts, particularly in endemic regions like Gombe, Nigeria. This study aimed to investigate the mechanisms underlying pyrethroid resistance and identify the prevalent Anopheles species in the area. Morphological identification was performed using keys from Gille and Coetzee, confirmed by molecular techniques employing SINE200 PCR for precise species characterization. The results revealed that the An. gambiae complex comprised 75% of the mosquito population, indicating its dominance in the region. Knockdown rate bioassays demonstrated a time-dependent increase in resistance to insecticides, with notable exceptions observed with deltamethrin. Susceptibility testing conducted 24 hours post-exposure confirmed that the population exhibited resistance to all tested insecticides, with DDT showing the highest resistance level. Molecular analysis identified Anopheles coluzzii as the most prevalent species in Gombe, followed by An. arabiensis. Additionally, the prevalence of kdr alleles was assessed, revealing a significant correlation between the L1014F mutation and resistance phenotypes. Specifically, the frequency of the L1014F allele was linked to increased resistance levels, while the homozygous susceptible allele was also

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prevalent, suggesting the potential influence of other resistance mechanisms. In conclusion, this study highlights the critical need for ongoing surveillance of insecticide resistance in Anopheles gambiae populations. It underscores the importance of understanding the genetic basis of resistance to inform effective vector control strategies. The findings emphasize that adaptive management of insecticide use, considering the dynamics of resistance and species composition, is essential for enhancing malaria control efforts in Gombe and similar regions.

Keywords: Pyrethroid Insecticides, Resistance, Susceptibility, PCR, Malaria

INTRODUCTION

Malaria remains one of the deadliest diseases worldwide, responsible for over 400,000 deaths annually, with 90% of these fatalities occurring among children under the age of five in Sub-Saharan Africa (WHO, 2017). Among the African nations, Nigeria bears the heaviest burden, contributing 27% of the global malaria cases (Access, 2018). Gombe State, located in north-eastern Nigeria, is endemic to malaria, registering a significant number of cases every year (USAID, 2015). Although ongoing malaria control efforts have made strides in reducing this burden, the persistence of mosquito breeding habitats, particularly in areas with rice fields and other agricultural activities, continues to fuel malaria transmission (Jabir et al., 2014).

Anopheles mosquitoes, particularly *Anopheles gambiae* sensu lato, serve as the primary vectors for malaria in the region (Adedayo et al., 2019). However, the widespread use of insecticides in both agriculture and public health interventions, such as Indoor Residual Spraying (IRS) and Long-Lasting Insecticidal Nets (LLINs), has led to increasing insecticide resistance among these vectors (Riveron et al., 2015). This resistance poses a significant threat to malaria control programs, as it undermines the effectiveness of vector control strategies, putting millions at risk of infection and death. Pyrethroid insecticides, the only class approved for mosquito net impregnation by the World Health Organization, have become a cornerstone of malaria prevention. However, the emergence of resistance to pyrethroids in *Anopheles gambiae* populations in Gombe raises concerns about the long-term viability of current malaria control measures (Chaumeau et al., 2017).

Understanding the mechanisms behind this resistance is critical to developing new strategies for malaria vector management. This study aims to investigate the biochemical and genetic mechanisms of pyrethroid resistance in *Anopheles gambiae* from Gombe, Nigeria. By identifying the specific resistance pathways, this research will provide valuable insights to guide future malaria control efforts and mitigate the impact of insecticide resistance in the region and beyond.

MATERIALS AND METHODS

Mosquito Sample Collection

Blood-fed female Anopheles mosquitoes were collected in April 2021 between 05:00 and 06:00 a.m. Random collection occurred from the ceilings and walls of various houses in Dadin-Kowa village, Gombe Local Government Area, using a battery-powered aspirator and a mouth aspirator.

Mosquito Sample Rearing

Collected mosquitoes were preserved in a mosquito rearing cage and fed with a 10% sucrose solution. Cotton wool soaked in the sucrose solution was placed inside the cage for the mosquitoes to feed. The female mosquitoes were then encouraged to lay eggs in 1.5-ml Eppendorf tubes. After hatching, the larvae were transferred to disposable cups containing water and later preserved in a rubber bucket in the insectary. They were maintained at room temperature with suitable humidity and fed with Tetramin baby fish food. Adults that emerged within 1-4 days were used for bioassays, while some were frozen for subsequent identification, biochemical analysis, and other experiments.

Insecticidal Bioassay

Insecticidal bioassays were conducted to measure the biological activity of pyrethroid insecticides on Anopheles mosquitoes, following WHO susceptibility test protocols (Tomlinson et al., 2019). Mosquitoes were exposed to insecticide-treated surfaces for 60 minutes. The number of knockdowns was recorded after 15, 30, 45, and 60 minutes. After exposure, mosquitoes were provided with a 10% sucrose solution in holding tubes. Mortality was assessed 24 hours post-exposure, and active mosquitoes were recorded. Samples were then stored in a refrigerator for later analysis.

Insecticide susceptibility bioassays

The World Health Organization developed a technique called the Insecticide susceptibility bioassay to track insecticide resistance in mosquito populations. The decade of the 1960s saw the early development of this technique, which has subsequently undergone numerous modifications. The collection of wild female adult mosquitoes or wild larvae, which are subsequently raised to adulthood in a test facility, is the foundation for resistance monitoring using this method. They are subsequently exposed to an insecticide at a discriminating concentration (DC) on filter paper that has been treated in order to measure their knockdown and mortality. The WHO bottle bioassay measures mosquito mortality after 1 hour of exposure to a known standard concentration (for example, the discriminating concentration) of an insecticide. It is a direct response-to-exposure test. At 24 hours (or 72 hours) following the 1-hour exposure period, the mortality of test mosquitoes is noted.

Morphological Species Identification

Morphological identification of *Anopheles gambiae sensu lato* was performed using Gille and Coetzee's (1987) keys. Specimens were examined under a stereomicroscope, focusing on distinctive features such as wing patterns, legs, tarsi, abdomen, palps, and veins.

DNA Extraction

Genomic DNA was extracted from both resistant and susceptible Anopheles mosquitoes using the Da AN gene nucleic acid extraction protocol. This involved adding lysis buffer, Proteinase K, and ground mosquito specimens into a microcentrifuge tube, followed by a series of centrifugation and incubation steps at specific temperatures. The extracted nucleic acid was stored for subsequent PCR analyses.

Molecular Species Identification

Molecular identification of the *An. gambiae* complex was conducted using the SINE PCR method (Santolamazza *et al*., 2008). Specific primers (SINE 200-F and SINE 200-R) were utilized for amplification.

PCR and Thermocycling Conditions

Thermocycling conditions included initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 60 seconds, and extension at 72ºC for 1 minute, concluding with a final extension at 72ºC for 5 minutes.

Amplified products were verified via electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Detection of Knockdown Resistance (kdr) L1014F Mutation

The kdr L1014F mutation was detected following the methodology of Martinez-Torrez *et al.* (1998). PCR conditions were similar to those used for species identification, and the presence of the mutation was confirmed through gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gels (1.5%) were prepared for visualizing PCR products. The gel was run in an electrophoresis machine under specified conditions to separate DNA samples based on size. DNA ladders were used as size markers during analysis.

RESULTS

Morphological specie identification

The data on morphologically identified mosquitoes used for insecticidal bioassays is displayed in **Table 1**. Identification was performed using specific morphological keys, which included characteristics such as the possession of speckled, sparsely patterned legs, abdominal segments lacking laterally projecting tufts of scales, and the hind tarsi 4 and 5 not being entirely pale. Additionally, the palps exhibited three pale bands, with the third main dark area of vein 1 featuring a pale interruption fused with the preceding pale spot. The scaling on the abdomen was notably very scanty. The results indicate that a significant majority of the Anopheles mosquitoes collected from the study sites belong to the *An. gambiae* complex, with a frequency of 75%. The remaining 25% were classified as other Anopheles species, highlighting the predominance of the *An. gambiae* complex in these regions.

Table 1. Morphologically observed species of anopheles mosquitoes in the study sites

Serial Number	Species	Number of sample (n)	Frequency (%)
1	*An. gambiae* complex	35	75
2	Other Anopheles	15	25

Knockdown rate bioassay result

The knockdown (KD) rate recorded after 60 minutes of insecticide exposure is illustrated in **Figure 1**. The results indicate that the effects of insecticide exposure on the mosquitoes were time-dependent, with an observable increase in the number of knockdowns as exposure time progressed from 0 to 60 minutes. Notably, an exception was observed with deltamethrin, where the KD rate at 15 minutes was higher than that recorded at 30 minutes. However, from 30 minutes to 60 minutes, the trend aligned with the overall pattern of increasing knockdown rates for all insecticides tested against the *Anopheles gambiae* population.

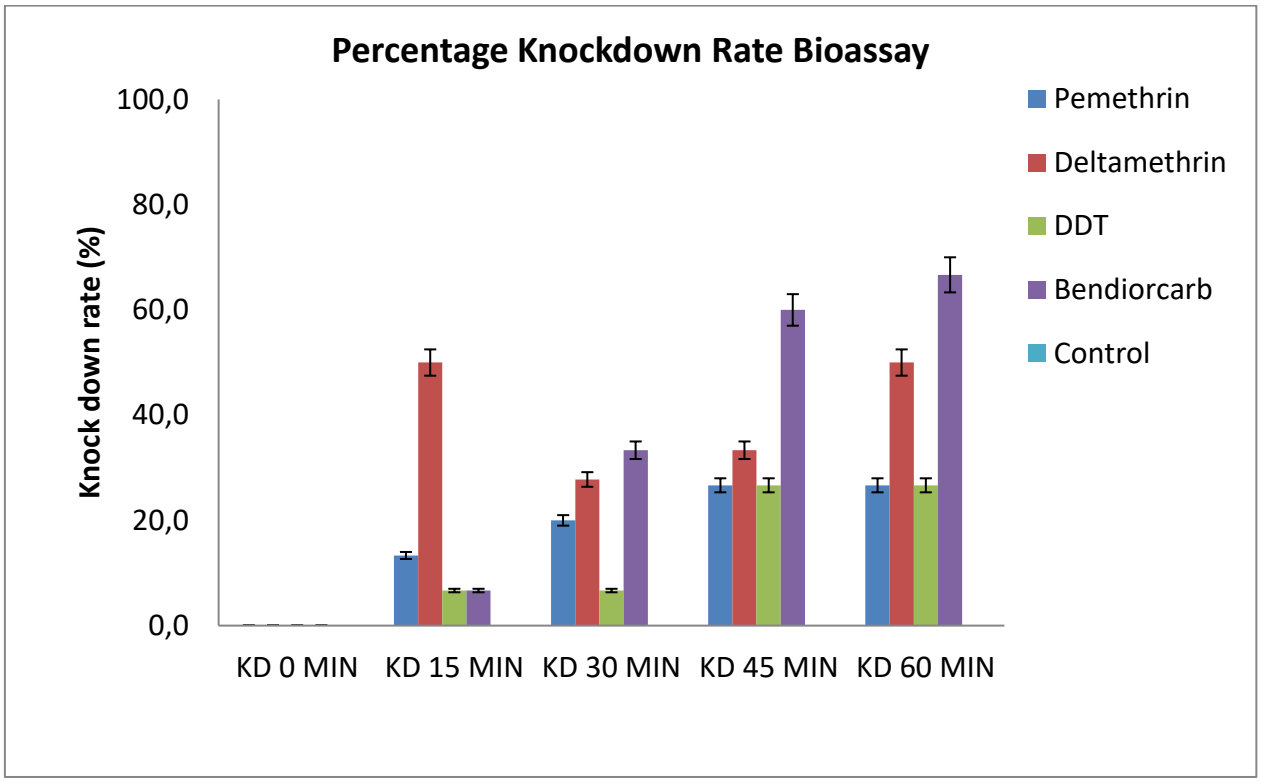


Figure 1 Knockdown bioassay using various public health pesticides. Results represent the average of percentage mortalities at 0, 15, 30, 45, and 60 minutes

following exposure from the Anopheles gambiae population in Gombe. Standard deviation of means is shown by error bars.

Activities of the Insecticides Against Anopheles gambiae After 24 Hours Post Exposure

Figure 2 illustrates the susceptibility status of the *An. gambiae* population treated with permethrin, deltamethrin, DDT, and bendiocarb. The results clearly indicate that the species exhibits resistance to all four insecticides tested. Among the pyrethroids, DDT demonstrated the highest resistance, with only 35% mortality observed. The population showed greater resistance ($p < 0.05$) to permethrin (40%) compared to deltamethrin (60%). Notably, the lowest resistance was recorded after exposure to bendiocarb, suggesting that this insecticide may remain effective against the *An. gambiae* population in the area.

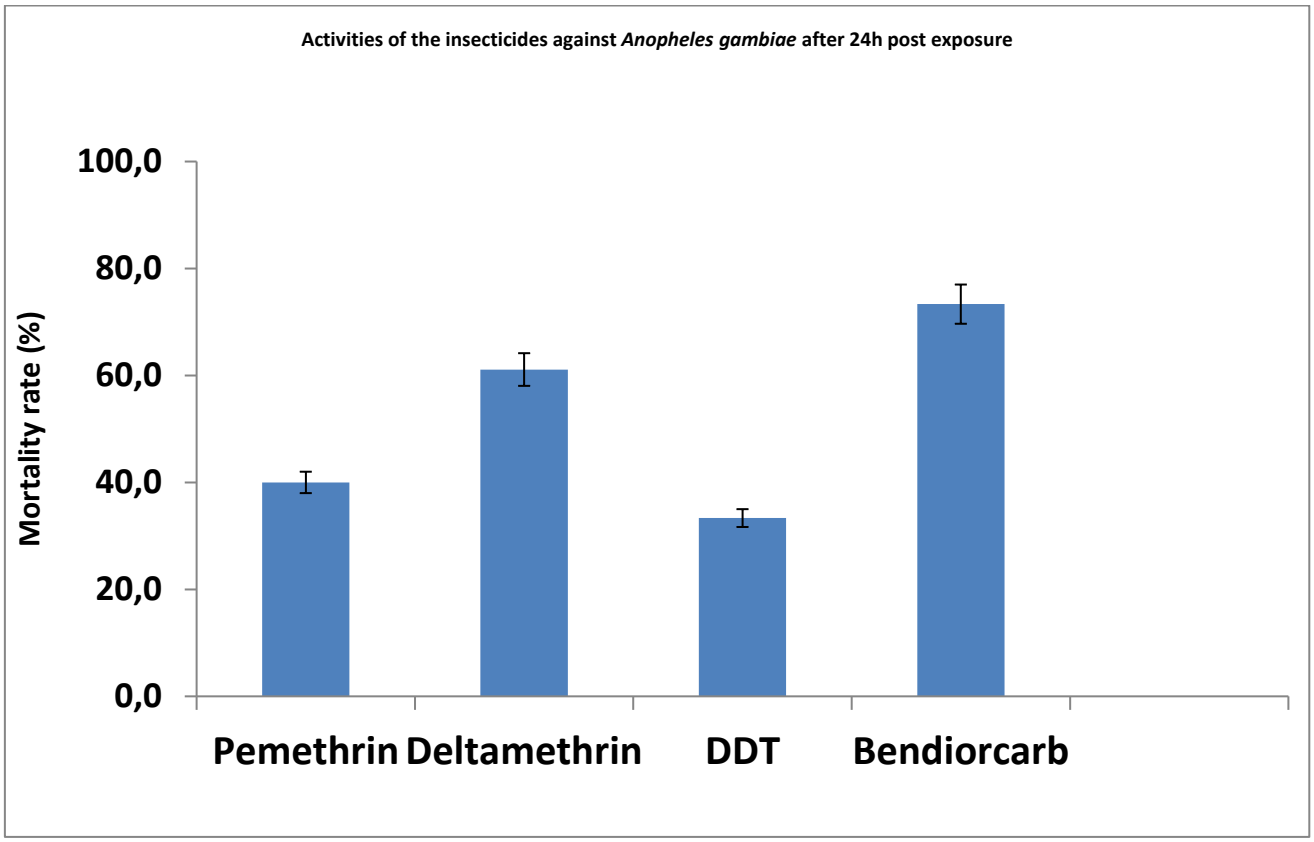


Figure 2. WHO tubes bioassays with different public health insecticides. Results are average of percentage mortalities at 24 h post-exposure from the Gombe population of Anopheles gambiae. Error bars indicate standard deviation of means.

Molecular Identification of Anopheles gambiae Sibling Species Using SINE200 PCR

The molecular identification of *An. gambiae* complex using SINE 200 PCR (Table 2) indicates that *An. coluzzi* was dormant species with a frequency of 92.86% (n=13). Only one *An. arabiensis* (n=1) was found in the sample tested. The respective bands representing the mosquito species are shown in Figure 3 which represents the results of agarose gel electrophoresis showing the bands observed at approximately 479 bp and 220 bp following the amplification of the short-interspersed nuclear element 200 (SINE200) for species identification of *An. gambiae* sibling species. Left Side Gel Picture: Lanes 1-10 exhibit a prominent band at ~479 bp, indicating the presence of *An. coluzzii*. Right Side Gel Picture: Lanes 1, 2, and 4 show bands at ~479 bp, confirming the identification of *An. coluzzii*. Lane 3 reveals a band at ~220 bp, indicating the presence of *An. arabiensis*. Lane 5 serves as a negative control, indicating no amplification. M represents the hyperladder IV DNA ladder [Bioline 100–1013 bp (40–200 ng/band)], which is used for size calibration. These results highlight the efficacy of the SINE200 PCR method in distinguishing between sibling species within the Anopheles gambiae complex, providing valuable insights for vector control efforts.

Table 2 SINE200 PCR for molecular specie identification of *A. gambiae s.l.*

Serial Number	Anopheles species	Number of sample (n)	Frequency (%)
1	*An. coluzzi*	13	92.86
2	*An. arabiensis*	1	7.14

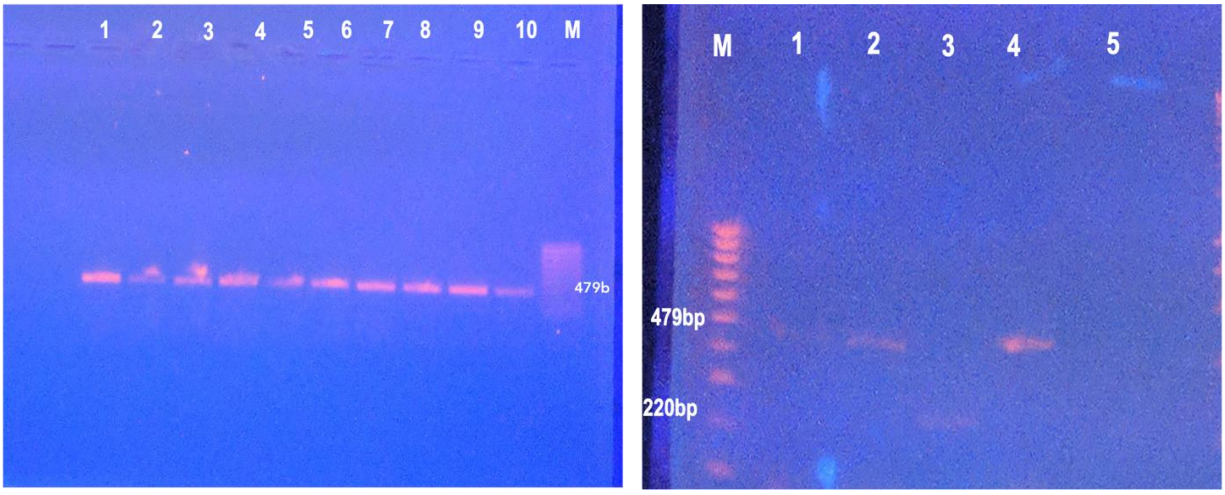


Figure 3: Agarose gel electrophoresis picture showing the bands observed at ~479 and 220 bp after amplification of the fragment of short-interspersed nuclear element

200 (SINE200) for species identification of An. gambiae sibling species. On a left side gel picture, lane 1 – 10 indicated the band at ~479 bp while on the right side gel picture lane 1,2, & 4 were bands at ~479 bp. Lane 3 revealed 220 bp. Band size band at ~479 bp represent An. coluzzii while 220 bp represent An. arabiensis. Lane 5 on the right side gel picture is a negative control. M is hyperladder IV DNA ladder [Bioline 100–1013 bp (40–200 ng/band)].

Prevalence of Kdr mutations in An. gambiae

To understand the resistance mechanism in the resistant *An. gambiae* population in Gombe, a malaria endemic region, the prevalence of *kdr* alleles and their correlation with observed resistance phenotypes were determined. The *Kdr* genotypes and frequency of alleles in tested *An. gambiae* are displayed in **Table 3** and **Figure 4**. The kdr genotypes include the homozygous L1014 (LL), the heterozygous L1014F (LF), and the homozygous F1014 (FF) mutations. The mosquitoes comprise phenotypically type resistant (Alive) and susceptible (dead). The resistant strains are represented by lane 1-7 on figure 4 while the susceptible strains are represented by lane 8-14 on the same figure. This PCR diagnostic test entails utilizing four oligonucleotides or primers (Agd1, Agd2, Agd3, Agd4) and a Taq polymerase to amplify a DNA fragment encoding the voltage-dependent sodium channel in each tested mosquito in order to look for resistant or vulnerable alleles. The kdr gene is flanked by the Agd1/Agd2 primer pair, which amplifies a 293 bp product as a checkpoint. The band labeled 293 on the hyperladder and its corresponding bands on all the lanes (1-14) in figure 4 represents the control. Only the resistance region of the KDR gene couples with the pair of Agd3/Agd1 primers, amplifying a 195 bp fragment that corresponds to the resistant allele. The band labeled 231 on the hyperladder and its corresponding bands in figure 4 represents the resistant allele. The Agd4/Agd2 pair amplifies a 137 bp segment that corresponds to the susceptible allele and is the sensitive region of the gene. The band labeled 137 on the hyperladder and its corresponding bands in figure 4 represents the susceptible allele. The nucleotide sequences of these primers are as follows: Agd1: 5'-ATA GAT TCC CCG ACC ATG -3'; Agd2: 5'-ACA AGG ATG ATG AACC- 3'; Agd3: 5'-AAT TTG CAT TAC TTA CGA CA-3'; Agd4: 5'-CTG TAG TGA TAG GAA ATT TA-3' (Martinez-Torres *et al.,* 1998)

A significant difference is observed in the distribution of the *L1014F* allele ($p < 0.05$) between resistant (28.571%) and susceptible (0%) phenotypes. Additionally, the frequency of resistant (28.571%) heterozygous allele (L1014F) is significantly different ($p < 0.05$)

compared to homozygous resistant (0%) allele (1014F/1014F) in resistant mosquito. However, the frequency (71.429%) of homozygous susceptible allele (1014L/1014L) is significantly higher than that of the heterozygous allele (L1014F). The findings shown that a heterozygous resistant allele (L1014F) on a DNA fragment encoding for the voltage-dependent sodium may contribute to the resistance to pyrethroid insecticides.

Table 3 Correlation between the L1014F allele frequency and resistance to pyrethroid (Deltamethrin/Permethrin) in *An. gambiae* populations

Phenotype		No. of L1014F alleles			Freq L1014F alleles (%)			Freq of T and L	
	N	LL	LF	FF	LL	LF	FF	Total L	Total F
Alive	7	5	2	0	71.429	28.571	0	12	2
Dead	7	7	0	0	100	0	0	14	0

Alive = resistant, Dead = susceptible, Freq= frequency, N= number of samples successfully genotyped, LL= homozygous susceptible genotype, LF= heterozygous resistant genotype and FF= homozygous resistant genotype, 95 % CI= 95 % confidence interval N= number of samples successfully genotyped

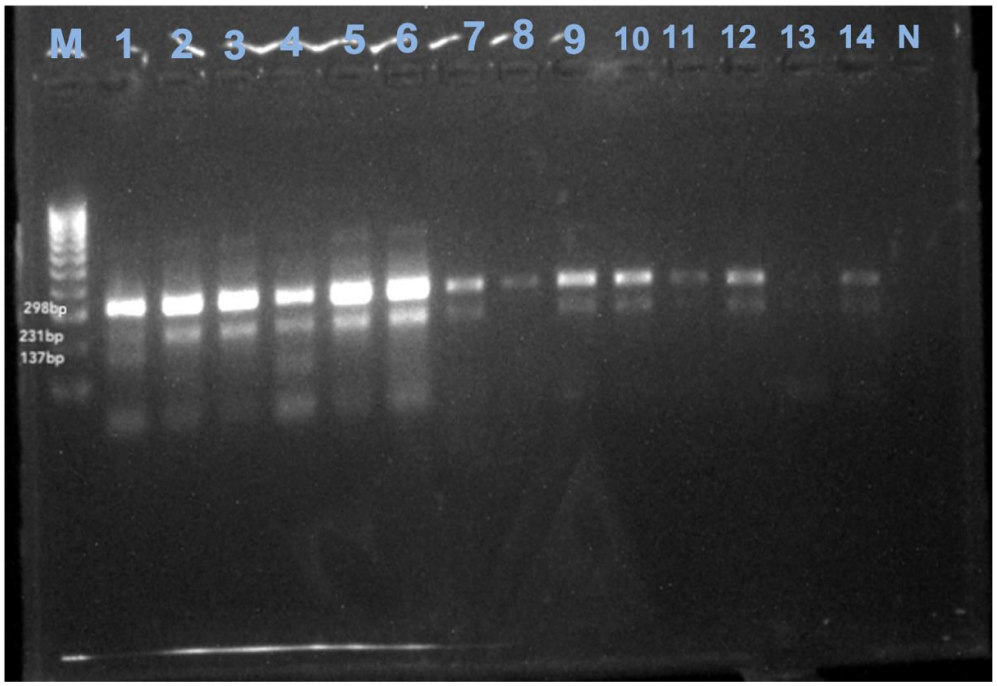


Figure 4. Agarose gel of allele specific PCR genotyping of L1014F mutation in adult population of An. gambiae from Gombe. Lane 1-7 are representative of resistant

and Lane 8-14 susceptible strains of An. gambiae. Lane 15 is a negative control; M is hyperladder IV DNA ladder [Bioline 100–1000bp].

DISCUSSION

The present study aimed to understand the mechanisms of pyrethroid resistance in the human malaria vector, *Anopheles gambiae* sensu lato. To achieve this primary objective, we identified the Anopheles mosquito species both morphologically and molecularly using the SINE200 PCR method.

Morphological identification was conducted based on keys described by Gille and Coetzee (1986). The results presented in Table 1 demonstrate that the *An. gambiae* complex has the highest frequency at 75%, significantly more than other species. This high frequency may be attributed to environmental conditions that favor the rapid reproduction of these mosquitoes. Consequently, the *An. gambiae* complex should be the primary target for monitoring malaria and other mosquito-borne diseases in Gombe. Olatunbosun-Oduola et al. (2019) also reported a high frequency of the An. gambiae complex in this region.

The knockdown (KD) rate, shown in Figure 1, was recorded at 0, 15-, 30-, 45-, and 60-minutes post-insecticide exposure, revealing a time-dependent effect. An anomaly was observed with deltamethrin at 15 minutes, where the KD value was unexpectedly higher than at 30 minutes. This unusual outcome may be attributed to physical stress experienced by the mosquitoes after being transferred to the exposure tubes, which could have affected their behavior and recovery.

The results of the 24-hour post-exposure insecticide susceptibility bioassay, illustrated in Figure 2, demonstrated resistance to all four insecticides tested, with the highest resistance observed for DDT. The population exhibited greater resistance ($p < 0.05$) to permethrin (40%) compared to deltamethrin (60%). The lowest resistance was recorded after exposure to bendiocarb. In contrast, *Anopheles gambiae* populations from Bichi (Kano, Northwest Nigeria) exhibited high levels of resistance to DDT and permethrin but lower levels of resistance to bendiocarb (Habibu *et al.*, 2017).

The observed disparities in resistance to these pyrethroids may stem from differences in their mechanisms of action. Yusuf *et al.* (2021) also reported resistance to deltamethrin, DDT, and bendiocarb in *An. gambiae* from Yamaltu Deba in Gombe, noting lower

knockdown rates compared to populations from Auyo (Jigawa State) and Kumbotso (Kano State). According to Toé *et al.* (2014), the increasing application of pesticide-based techniques for malaria vector control may contribute to the development of insecticide resistance. Olatunbosun-Oduola *et al.* (2019) similarly observed multiple pesticide resistance in *Anopheles gambiae s.l.* mosquitoes in eight communities in Southern Gombe.

The SINE200 PCR results (Figure 1) identified *Anopheles coluzzii* (M form) in Gombe, with the highest frequency (74%) among the *Anopheles gambiae* complex members. While *An. arabiensis* was less abundant, its presence indicates the coexistence of various Anopheles species in similar habitats. Wahedi *et al.* (2021) employed species-specific PCR and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) techniques to identify sibling species in exposed mosquitoes from diverse regions of Adamawa State, reporting that *An. coluzzii* constituted the majority surviving after exposure to deltamethrin, DDT, and bendiocarb (78.5–92%), in contrast to *An. gambiae* s.s. (5.3–11%) and *An. arabiensis* (0–10.1%). The dominance of *A. coluzzii* may be attributed to favorable environmental conditions for this species.

To comprehend the resistance mechanisms in the *An. gambiae* population in the malaria-endemic region of Gombe, we assessed the prevalence of kdr alleles and their relationship to resistance phenotypes. The frequencies of the L1014F, F1014F, and L1014L alleles in resistant mosquitoes were 28.571%, 0%, and 71.429%, respectively, as shown in Table 4.3. Although the L1014L allele has a significantly higher frequency compared to L1014F, the observed resistance is partly attributed to the presence of the L1014F allele. A positive correlation exists between the frequency of the L1014F mutation and the level of resistance, indicating that a higher frequency of this mutation correlates with increased resistance. Conversely, the homozygous resistant allele does not contribute to the observed resistance. The high frequency of the homozygous susceptible allele suggests the influence of other factors, such as metabolic resistance or additional mutations like the East African mutation (L1014S). Habibu *et al.* (2017) reported the co-occurrence of L1014S and L1014F mutations alongside significant pesticide resistance in two Anopheles populations from agricultural and residential settings in Northwest Nigeria, indicating that the L1014S mutation may be spreading throughout Africa.

In summary, this study highlights the complexities of pyrethroid resistance mechanisms in *An. gambiae* populations in Gombe, emphasizing the need for continuous monitoring and the development of targeted vector control strategies to combat malaria effectively.

CONCLUSION

This study provides valuable insights into the mechanisms of pyrethroid resistance in *Anopheles gambiae* sensu lato within the malaria-endemic region of Gombe, Nigeria. Our findings indicate that the *An. gambiae* complex predominates in this area, with a frequency of 75%, highlighting its significance as a target for malaria vector control efforts. The knockdown rate analysis revealed a time-dependent response to insecticide exposure, with notable resistance to deltamethrin and permethrin, as well as the highest resistance observed for DDT.

Molecular identification using SINE200 PCR confirmed the presence of *Anopheles coluzzii* and *Anopheles arabiensis*, indicating a diverse mosquito population in Gombe. The assessment of kdr alleles demonstrated a significant correlation between the L1014F mutation and resistance phenotypes, suggesting that this mutation plays a critical role in the observed resistance.

Overall, these findings underscore the urgent need for ongoing monitoring of insecticide resistance in *Anopheles gambiae* populations and the importance of adapting vector control strategies to mitigate the impact of malaria transmission in the region. Enhanced understanding of resistance mechanisms will aid in the development of effective interventions to combat malaria and other mosquito-borne diseases.

Competing interest

Authors declare that no competing interest exist.

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