Mosquito Species Composition And Insecticide Resistance Status of
Anopheles Arabiensis In Itang Special Woreda, Gambella, Southwestern
Ethiopia

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Research

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Abstract

Introduction: *Anopheles arabiensis*, member species of the *Anopheles gambiae* complex, is the primary vector of malaria widely distributed in Ethiopia. *Anopheles funestus, An. pharoensis* and *An. nili* are secondary vectors occurring with limited distribution in the country. Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are pillars for the interventions against malaria control and elimination efforts in Ethiopia. However, the emergence and widespread of insecticide resistance in the major malaria vector, *An. arabiensis*, might compromise the efforts of the country. The aim of this study was to investigate composition of mosquito species and insecticide resistance status of *An. arabiensis* in Itang special woreda (district), Gambella, southwestern Ethiopia.

Materials and methods: Adult mosquitoes were sampled from September 2020 to February 2021 using Centers for Disease Control and Prevention (CDC) light trap and Pyrethrum Spray Catch (PSC). Moreover, mosquito larvae were also collected from different breeding sites and reared to adults to assess susceptibility status of populations of *An. gambiae* s.l. in the study area. Susceptibility tests were conducted on two to three days old non blood fed female *An. gambiae* s.l using insecticide impregnated papers with deltamethrin (0.05%), alpha-cypermethrin (0.05%), propoxur (0.1%), pirimiphos-methyl (0.25%) and bendiocarb (0.1%) following World Health Organization (WHO) standard susceptibility test procedure. Molecular diagnostics were done for the identification of member species of *An. gambiae* s.l and detection of knockdown resistance (*kdr*) allele using species specific polymerase chain reaction (PCR) and allele specific PCR.

Results: In total, 468 adult mosquitoes were collected from different houses. *Culex* mosquitoes were the most dominant (80.4%) followed by *Anopheles* mosquitoes. Three species of *Anopheles* mosquitoes (*An. coustani, An. pharoensis*, and *An. gambiae* (s.l.)) were identified, of which *An. coustani* was the dominant (81.1%) species. WHO bioassay tests revealed that the populations of *An. gambiae* s.l in the study area are resistant against alpha-cypermethrin and deltamethrin whereas, susceptible to bendiocarb, pirimiphos-methyl and propoxur. Out of the total 86 *An. gambiae* s.l specimens assayed, 79 (92%) successfully amplified and identified as *An. arabiensis*. West African *Kdr* (L1014F) mutation was detected with high *Kdr* allele frequency ranging from 67-88%.

Conclusion: The detection of target site mutation, *kdr* L1014F allele, coupled with the phenotypic resistance against alpha-cypermethrin and deltamethrin call for continuous resistance monitoring.

Background

Malaria is vector-borne disease caused by *five Plasmodium* species namely, *Plasmodium falciparum, P. ovale, P. malariae, P. vivax and P. knowlesi*. The parasite transmitted to human through the bite of infective female *Anopheles* mosquito [1]. There are over 445 recognized species of *Anopheles* mosquito of which around 70 species are potential malaria vectors [2]. In Africa, the major malaria vectors are *Anopheles gambiae* and *An. funestus* species complexes, but there are also a number of primary and secondary vectors that contribute to the malaria transmission [2].

In Ethiopia, there are more than 45 documented *Anopheles* mosquito species [3], of which only four species are malaria vectors. *Anopheles arabiensis*, member species of the *An. gambiae* complex, is the primary vector of malaria widely distributed in the country [4, 5]. *Anopheles funestus* *An. pharoensis* and *An. nili* are secondary vectors occurring with varying population densities, limited distribution and vector competence [6]. Very recently, an invasive *Anopheles* species, *An. stephensi*, has been documented in the country [7] which might complicate the malaria elimination effort of the country [8].

Chemical based vector control intervention is the pillar strategy to combat malaria. Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are instrumental for the significant reduction of malaria morbidity and mortality [9]. However, the emergence and widespread of insecticide resistance in the major malaria vectors particularly in Africa might compromise effectiveness of chemical based (IRS and LLINs) interventions against malaria control and elimination efforts [9–15].

Insecticide resistance has been reported in more than 500 insect species worldwide among which over 70 *Anopheles* species (Diptera: Culicidae) are responsible for the transmission of malaria parasites to humans (Hemingway and Hilary, 2000). Insecticide resistance has become a serious challenge for the control and elimination of malaria due to the fact that malaria vectors are resistant to the four commonly used insecticide classes (pyrethroids, organochlorines, carbamates and organophosphates) [9, 15]. In Ethiopia, DDT resistance by *An. arabiensis* was reported in the 1990s, since then widespread DDT resistance was documented throughout the country [10, 14, 17–19]. Moreover, resistance against other classes of insecticides such as carbamates (bendiocarb), organophosphates (malathion) and pyrethroids (permethrin, deltamethrin) has been reported from various regions of the country [19, 20].

In the last decade the number of malaria cases has declined due to a high coverage of IRS and scaling up of LLINs [9, 15]. This result initiated the national malaria control and elimination program of Ethiopia to develop national malaria elimination road map to eliminate malaria from the country by 2030 [21]. However, this plan might be compromised as the magnitude of resistance against several insecticides is increasing in the *An. arabiensis* populations [9, 19, 20].

Insecticide resistance largely caused by two major mechanisms. The first one is due to target-site insensitivity as a result of mutations in the target site of the insecticide that changes binding. The second mechanism is metabolic based resistance, where the insecticide is either degraded, sequestered or transported/excreted out of the cell before binding to the target site [22, 23].
Target-site and metabolic based resistance mechanisms operating in malaria vectors in several malaria endemic African countries have been documented. Knockdown resistance (kdr) is target site mutations in the voltage-gated sodium channel gene of mosquito nerve membranes is associated with DDT and pyrethroids resistance. In *Anopheles*, this involves the substitution of leucine (TTA) to phenylalanine (TTT) (kdr.L1014F) or to serine (TCA) (kdr.L1014S) [24, 25]. In addition, substitution of asparagine to tyrosine (N1575Y) is linked with resistance in *An. gambiae* s.s [26] but not in *An. arabiensis* [19]. There is also an acetylcholinesterase gene (ace-1R) mutation, substitution of glycine (GGC) to a serine (AGC) which confers resistance to organophosphates and carbamates [27]. In Ethiopia, target site resistance mechanism, Kdr.L1014F (West Africa Kdr), in populations of *An. arabiensis* documented in several populations across the country [10, 11, 17, 19, 20]. Metabolic based resistance in *Anopheles* mosquitoes have been reported from several countries in Africa [28–31]. Moreover, modifications in the cuticle either through cuticle thickening and/or altering of the cuticle composition of arthropods which can slow down the penetration of chemical compounds [32–34] has also been reported in *Anopheles* populations [35].

Approximately 60% of the Ethiopian populations live in malaria risk areas [6]. The disease primarily occurs up to the 2000-meter (m) elevation but can also occasionally affect areas over 2000m elevation in response to the spatial and temporal changes [36, 37]. Malaria transmission is unstable and seasonal which produces little immunity in the community; hence malaria epidemics are common and lead to high mortality and morbidity [6]. Gambella is one of the malarious areas of Ethiopia with high malarial endemicity. Itang special woreda is known for its a stable form of malaria transmission [38]. Despite the current effort of the country, malaria incidence rate in Itang did not decline unlike many other malarious areas of Ethiopia. [39]. Moreover, to the best of our knowledge composition of mosquito species and insecticide resistance status of *Anopheles gambiae s.l* in Itang, not yet studied, and the resistance mechanisms operating in the populations not known. Therefore, this study aimed to investigate mosquito species composition, insecticide resistance status of *Anopheles gambiae s.l* and detection of target site mutations associated with DDT and pyrethroid resistance.

### Methods And Materials

#### Study area and mosquito collections

Gambella is administrative regional state located in south western Ethiopia 777 km away from Addis Ababa (Fig. 1). Most of the region is flat, hot, and humid, with an altitude range of 300–2,300m above sea level and sloping westward. The annual average temperature of the region is 21.1°C–35.9°C, with an average annual rainfall of 600 mm. The region has a total area of 25,802 km² and administratively divided into three Zones (Nuwer, Aguna, Mezeng) and one special woreda, Itang [40]. According to the 2017 Ethiopian population projection, Gambella is sparsely populated with the total population approximately 436,000 [41].

The study was conducted from September 2020-February 2021 in Itang special woreda, which is situated 42 km to the west of the regional capital of Gambella. Itang has 23 kebeles, with estimated total population of 45,772 and 9,154 households [41]. The woreda's average annual temperature and rainfall are 29°C and 1,000 mm, respectively. The climatic conditions of the woreda are favorable for the existence of a stable form of malaria throughout the year [6].

Potential mosquito breeding habitats (Baro river fringes, paddle or farming field ditches, sewerage, and stagnant water pools) were first inspected for the presence of mosquito larvae and positive habitats were sampled with a 350 ml capacity mosquito scoop. Dipping was done following WHO guidelines and standard operating procedures for entomological surveillances [42]. Anopheline mosquito larvae were sampled from breeding sites, and kept in a room at constant 80±10% RH and 27±2°C. The larvae were fed baking powder. The pupae were sorted and transferred to beakers and placed inside a cage adults to emerge. Adults were provided 10% sugar in the cage and, 2-3 days old female mosquitoes were used for insecticide susceptibility tests.

Adult *Anopheles* mosquitoes were collected using CDC light traps and Pyrethrum Spray Catch (PSC) from selected houses in study area. CDC light traps were placed in three selected houses indoor and outdoor from 6:00PM to 06:00AM to collect female *Anopheles* mosquitoes for two consecutive days per month from September 2020 to February 2021. In addition to CDC light traps, indoor resting mosquitoes were collected using PSC in ten selected houses from 6:00AM to 9:00AM once in a month from October 2020 to February 2021. Before spray the whole house was covered by white sheet and closed every opening such as windows, door, and others. After fifteen minute of spraying knockdown mosquitoes were collected and recorded and separately placed in Eppendorf tube. *Anopheles* mosquitoes were morphologically identified to the species using taxonomic keys [43, 44].

#### Insecticide susceptibility test

Two to three days old non blood fed adult females morphologically identified as *An. gambiae s.l* were exposed to insecticide impregnated papers with discriminating doses of deltamethrin (0.05%), alpha-cypermethrin (0.05%) propoxur (0.1%) pirimiphos-methyl (0.25%), bendiocarb (0.1%) and control papers impregnated with oil. Insecticides were selected based on their current operational significance in the national malaria control program. Pirimiphos-methyl, propoxur and bendiocarb are currently used for IRS in Ethiopia and pyrethroid is incorporated in LLINs. The insecticide impregnated and control papers were obtained from the WHO collaboration Centre, Vector Control Research Unit, School of Biological Sciences, Penang, Malaysia. The insecticides were first tested on susceptible strain *An. arabiensis* collected from Jimma University Tropical and Infectious Disease Research Center insectary to assure the quality of the impregnated papers. Then, the susceptibility test was carried out on nonblood fed female mosquitoes collected and reared from the study area. Batches of 25 mosquitoes in four replicates were exposed in test kit tubes with insecticide impregnated papers and a control in two replicates, each with equal number of mosquitoes, exposed to papers impregnated with silicone oil was run in parallel for all bioassays for one hour. Knockdown were recorded at 10, 15, 20, 30, 40, 50, and 60 minutes [45]. After one hour, mosquitoes were transferred into holding tubes and provided with 10% sucrose solution with soaked cotton pads. Mortality was recorded 24 hour post exposure. Mosquitoes, both dead and alive, were individually preserved in Eppendorf tubes over silica-gel for further molecular assays. The same numbers of mosquitoes were exposed to insecticide free papers as controls.
DNA extraction of An. gambiae s.l

Genomic DNA of individual Anopheles mosquito was extracted from 75 survived and 11 dead mosquitoes (sampled from mosquitoes exposed to alpha-cypermethrin and deltamethrin) using DNAzol reagent (MRCgene, USA) with minor modification of the protocol [46].

Molecular identification of An. gambiae s.l

Morphologically identified Anopheles gambiae s.l female mosquitoes (alpha-cypermethrin and deltamethrin survived and dead mosquitoes) were selected for the molecular identification of members of An. gambiae s.l species using species specific polymerase chain reaction (PCR) at Molecular Biology Laboratory, Tropical and Infectious Diseases Research Centre (TIDRC), Sekoru, Jimma University. An. arabiensis susceptible colony strain was used as a positive control. The PCR assay was done adapting the established protocol [47]. Finally, the band size of PCR products for each species was visualized on a 2% agarose gel.

Detection of LF14F (Kdr allele)

Allele specific PCR assay was conducted on the same specimens used for the identification of member species of An. gambiae s.l. as indicated under 3.5 above. The presence of West Africa Kdr (L1014F) mutation was detected adapting the established protocol [24]. Finally, the band size of the PCR products for kdr allele was visualized on a 2% agarose gel to determine the genotype to homozygous resistant (RR), heterozygous resistant (RS) and susceptible or wild type (SS) Kdr allele.

Data analysis

Susceptibility status data was analyzed based on the WHO 2016 classification criteria. As per the criteria 24 hour mortality rate 98% and above considered fully susceptible; between 90 and 98%, possible resistance or suspected resistance; and mortality below 90% classified as resistant. When the control mortality was between 5 and 20%, the average observed mortality was corrected using Abbott’s formula [48]. When the control mortality was above 20% the test result was discarded and the test was repeated.

Results

Mosquito densities and species composition

In total, 468 mosquitoes were collected using CDC light trap and PSC collection methods from different houses (Table 1). The majority of mosquitoes were Culex spp. (80.4%) followed by Anopheles mosquitoes. Three species of Anopheles mosquitoes such as An. coustani, An. pharoensis, and An. gambiae s.l were identified, of which An. coustani was dominant among others (8.1%; n=38) (Table 1). As shown in Table 1 the higher number of mosquito was collected outdoor by CDC light traps.

Table 1

| Mosquito Species | Light trap | PSC | Total |
|-----------------|------------|-----|-------|
|                 | Indoor (%) | Out door (%) | (%) |
| An. Coustani    | 10         | 5.4 | 26    | 11.2 |
| An. Pharoensis  | 8          | 4.3 | 24    | 10.4 |
| An. gambiae s.l | 9          | 4.8 | 8     | 3.5  |
| Culex spp       | 159        | 85.5 | 173  | 74.9 |
| Total           | 186        | 100 | 231   | 100  |

Insecticide resistance status of An. gambiae s.l against different insecticides

As per WHO criterion, the local mosquito populations of An. gambiae s.l. were resistant to two groups of pyrethroid insecticides (deltamethrin and alpha-cypermethrin). Mortality rates of An. gambiae s.l against deltamethrin and alpha-cypermethrin was 58% and 42%, respectively. However, the populations of An. gambiae s.l. were completely susceptible to pirimiphos-methyl, propoxur and bendiocarb, where 100% mortality rate was recorded for the three insecticides (Table 2).
studies from different parts of Ethiopia where propoxur fully killed bendiocarb has been developed by from different regions in Ethiopia. The population of An. gambiae s.l. in Ethiopia reported very high resistance to pyrethroids but susceptible to bendiocarb, propoxur and pirimiphos-methyl. In contrary, populations of An. gambiae from different localities showed that they were highly resistant to deltamethrin like other populations of An. gambiae found in other parts of Ethiopia. The molecular identification of member species of Anopheles gambiae s.l. showed that the presence of the knock-down resistance (kdr) allele frequency ranging from 67-88% (Table 3).

Table 2

| S.N | Insecticide        | An. gambiae s.l tested | Phenotypic resistance |
|-----|-------------------|------------------------|-----------------------|
|     |                   | No. tested | No. dead | % mortality |
| 1   | Alpha-cypermethrin (0.05%) | 100        | 42       | 42%         | Resistant |
| 2   | Deltamethrin (0.05%)     | 100        | 58       | 58%         | Resistant |
| 3   | Propoxur (0.1%)          | 100        | 100      | 100%        | Susceptible |
| 4   | Bendiocarb (0.1%)        | 100        | 100      | 100%        | Susceptible |
| 5   | Pirimiphos-methyl (0.25%)| 100        | 100      | 100%        | Susceptible |

Molecular identification of An. gambiae s.l and detection of L1014F Kdr allele

Out of the total 86 An. gambiae s.l specimen assayed using species specific PCR, 79 (92%) of the specimens were successfully amplified and all were identified as An. arabiensis. The allele specific PCR revealed the presence of the knock-down resistance (kdr) allele frequency ranging from 67-88% (n=51) homozygous and 7.3% (n=4) heterozygous kdr resistance allele respectively with the Kdr allele frequency ranging from 67-88% (Table 3).

Table 3

| Insecticide | Number assayed | Bioassay phenotype | Genotype | Allele frequency |
|-------------|----------------|--------------------|----------|------------------|
|             |                |                    | SS       | RS               |
| Deltamethrin| 34             | Survived           | 5 4 16   | 72 28            |
|             | 6              | Dead               | 1 - 2    | 67 33            |
| Alpha-cypermethrin | 41        | Survived           | 4 - 30   | 88 12            |
|             | 5              | Dead               | 1 - 3    | 75 25            |

Note: RR-Homozygous resistant, RS-Heterozygous resistant and SS-homozygous susceptible, R-resistant, S-wild type

Discussion

Anopheles coustani, An. pharoensis and An. gambiae s.l. were the three species identified from Itang, southwestern Ethiopia. Of all Anopheles mosquitoes, An. coustani was the most prominent species followed by An. pharoensis and An. gambiae s.l. In the study site the abundance of mosquito species collected by CDC light trap was higher outdoor than indoor. Unlike many other localities in Ethiopia, the abundance of An. coustani in Itang special woreda, southwestern Ethiopia, was dominated by An. gambiae s.l. A study from Lare, Gambella documented higher density of An. gambiae s.l than An. coustani contrasting the result of the current study. This difference might be due to sampling period differences which leads to variation or shifts in density for various species of mosquitoes. Taye and his coworkers sampled mosquitoes from May to September 2017 whereas in our study sampling time was between September and February. Moreover, the difference might also be due to the type of breeding habitat. The breeding site where the Anopheles mosquitoes sampled was shore to the Baro River and due to this reason the breeding sites were covered by plants and shaded the water which might be favorable for An. coustani. Very recently, higher density of An. coustani than An. gambiae s.l populations were reported from non-irrigated swampy and river edges in Arjo Didessa, South west Ethiopia. Unlike An. coustani, An. gambiae s.l. typically breeds in small, clear, sunlit temporary water pools where vegetative cover is low. Anopheles gambiae s.l populations from the study area were detected of Plasmodium species showing evidence for the malaria transmission in the country. Therefore, the high abundance of An. coustani in Itang might call attention to further study its role in malaria transmission.

The molecular identification of member species of An. gambiae s.l confirmed that An. arabiensis is the only member species found in the study area. This finding is similar to previously reported studies from other parts of Ethiopia. Anopheles arabiensis populations from the study area were found highly resistant to deltamethrin like other populations of An. arabiensis from different localities in Sudan and Uganda. The current study also showed that An. arabiensis populations were resistance to alpha-cypermethrin. Similarly, studies from South-West Ethiopia and Congo reported very high resistance to pyrethroids but susceptible to bendiocarb, propoxur and pirimiphos-methyl. In contrary, populations of An. arabiensis form Malawi were found susceptible to alpha-cypermethrin.

The population of An. arabiensis collected from Itang found fully susceptible to bendiocarb and propoxur. This report is in agreement with other studies from different regions in Ethiopia, Sudan, Yemen, Burkina Faso and Chad. However, unlike the current finding resistance against bendiocarb has been developed by An. arabiensis populations in some other parts of Ethiopia. The current result is similar to previously reported studies from different parts of Ethiopia where propoxur fully killed An. gambiae s.l. However, in some other parts of Ethiopia...
resistance against propoxur has been observed [19, 20]. Similar to propoxur and bendiocarb the An. arabiensis populations from the study area were found susceptible to pirimiphos-methyl. This is similar with studies from different parts of Ethiopia [11, 19], Uganda [68] and Congo [62].

In this study, the population of An. arabiensis were screened for West African kdr allele (L1014F). A very high frequency of the West African kdr allele (L1014F), was observed with kdr allele frequency ranging from 67-88% indicating that the target site resistance mechanism (Kdr) might contributed for the observed high level of pyrethroid (deltamethrin and alpha-cypermethrin) resistance in the population. This is similar with populations of An. arabiensis in some other areas of Ethiopia [11, 17, 19, 20]. The mutation, L1014F, is widespread in West and West Central Africa [69, 70] also becoming common in East African countries including Sudan [71], Tanzania [72, 73] and Kenya [74, 75]. The L1014F kdr mutation in the voltage gated sodium channel is a target point mutation against pyrethroids and DDT [25, 76] which might occurred by natural selection or by the use long and extensive use of DDT and pyrethroids for ITNs.

LLINs and IRS are the two most widely implemented malaria vector control interventions, and have resulted in a significant reduction of malaria-related mortality and morbidity in Ethiopia [9, 39, 77]. Universal access and coverage of LLINs by all household members regardless of age or gender [78] is not yet achieved in Ethiopia [78–80].

Conclusion And Recommendation

Overall, this study demonstrated that An. coustani was the predominant Anopheles species recorded among adult Anopheles mosquito collected during the study period in the study area. The high abundance of the species in the study area might call attention to further study the species for its role in malaria transmission. It also revealed that An. arabiensis, a member of An. gambiae complex, has developed high level of resistance against deltamethrin and alpha-cypermethrin. Moreover, target site mutation L1014F kdr allele with high frequency was detected in the populations.

For the success of malaria elimination from Ethiopia by 2030 [6], it is important to control the malaria vectors and focus on successful treatment. Therefore, understanding the type and density of malaria vector species in specific localities, their feeding behavior and interaction with humans is crucial for effective malaria vector control strategies. Moreover, evidence based insecticide susceptibility status of the malaria vector populations and understanding mechanisms of resistance operating in the populations is key for effective insecticide resistance management.

Abbreviations

CCEs: Carboxylcholinesterases

CDC: Center for Diseases Control and Prevention

CSA: Central Statistics Authority

DDT: Dichlorodiphenyltrichloroethane

DNA: Deoxyribonucleic Acid

FMoH: Federal Minister of Health

GSTs: Glutathione S-transferases

ITNs: insecticide treated nets

Kdr: Knock down resistance

KM: kilometer

LLINs: Long-Lasting Insecticide Treated Nets

MSF: Medicines sans frontiers

OCs: Organochlorines

Ops: Organophosphates

P450s: Cytochrome-P450 monoxygenase

PSC: Pyrethrum Spray Catches

PY: Pyrethroids

SPSS: Statistical package for the social sciences

VGSC: Voltage gated sodium channel
WHO: World Health Organization

**Declarations**

**Ethics approval and consent to participate**

Ethical approval letter (Ref. No. RPG/056/2020) was obtained from research and ethical review board of College of Natural Sciences, Jimma University, Ethiopia.

**Consent for publication**

Not applicable

**Availability of data and materials**

Data used for this study are included in the manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

TC, DY and EA conceived and designed the study. TC performed the field and laboratory experiments and analyzed data and drafted the manuscript. GN drafted the manuscript. DA developed the map of the study site. DY and EA analyzed and interpreted and critically reviewed the manuscript. All authors read and approved the final manuscript.

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**Figures**

*Figure 1*

Map of the study area