Identification of main malaria vector species and their sensitivity to insecticides used for malaria control and Long Lasting Insecticide-treated Nets (LLINs) efficacy in the Democratic Republic of Congo (DRC)

MSF-OCA, Mweso, North Kivu, DRC
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1. List of abbreviations
ANC Antenatal Care
CDC Centre for Disease Control, USA
DRC Democratic Republic of Congo
EIR Entomological Inoculation Rate
ELISA Enzyme-Linked Immuno Sorbent Assay
IDP Internally Displaced People
IRS Indoor Residual Spraying
Kdr Knock-down resistance
LLIN Long Lasting Insecticide-treated Net
MSF Médecins Sans Frontières
OPD Out-Patient Department
PCR Polymerase Chain Reaction
WHO World Health Organisation
WHOPES World Health Organisation Pesticide Evaluation Scheme

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4. Summary

Background
Malaria is an ever-increasing public health problem in the Democratic Republic of Congo (DRC) and is compounded by recurrent conflicts in the country. In 2012, MSF initiated research studies on malaria to understand the unprecedented incidence of malaria, including studies on adherence and efficacy of treatment, coverage of bed nets and their use, and malaria vector susceptibility to insecticides. The ultimate aim is to better target available interventions. We report on the main vector species involved in malaria transmission and their susceptibility towards insecticides, and on the efficacy of Long Lasting Insecticide-treated Nets (LLINs) in Kashuga and Mweso, North Kivu in 2017.

Methods
Mosquitoes were collected from 269 houses between January and April 2017 using CDC light traps. Via the maternity department of the health center in Kashuga, 32 LLINs of five different brands were collected (PermaNet 2.0=11; DuraNet=5; Netprotect=1; Olyset=2; Yorkool=13). Standardized WHO insecticide susceptibility tests (DDT, bendiocarb, α-cypermethrin, permethrin, deltamethrin, malathion and pirimiphos-methyl) and a CDC bottle bioassay (α-cypermethrin) were performed using malaria vectors collected from local breeding sites in Kashuga and Mweso. Different sides of new LLINs (PermaNet 3.0, Olyset plus, Netprotect) were tested by using standardized WHO cone bioassays with local malaria vectors. In the laboratory, mosquito species determination and the detection of the knock-down-resistance (kdr) mutation in these mosquitoes was done by PCR. An ELISA was performed to determine Plasmodium falciparum infection rates. The effectiveness of used LLIN was tested by WHO cone bioassays with the laboratory reared malaria vector Anopheles coluzzii.

Results
In total, 324 malaria vectors were collected. All were Anopheles gambiae s.l.. These mosquitoes showed a high P. falciparum sporozoite infection rate of 13.9%. The mutations kdr-east and kdr-west, were both present in the local vectors. The insecticide susceptibility tests performed with mosquitoes from Kashuga and Mweso showed resistance for DDT and all pyrethroids tested. In Kashuga the mortality for DDT was 21.2% and for the pyrethroids deltamethrin, α-cypermethrin and permethrin the mortalities were 65.6%, 50% and 9.2%, respectively. In Mweso the mortality for deltamethrin and α-cypermethrin were respectively 54.9% and 34.4%. DDT and permethrin were not tested with mosquitoes collected in Mweso. The carbamate bendiocarb and the organophosphates pirimiphos-methyl and malathion showed to be effective towards local malaria vectors resulting in 100% mortality, except for mosquitoes from Mweso exposed to pirimiphos-methyl (72.3%). The effectiveness of new LLINs on local malaria vectors varied. The PermaNet 3.0 with PBO, resulted in the highest mortality, 100% for mosquitoes from Kashuga and 95% for mosquitoes from Mweso. The Olyset plus net with PBO resulted in a higher knock-down than mortality, however, the mortality was still higher than mortality caused by nets without PBO. The effectiveness of used LLINs on non-resistant mosquitoes from a laboratory strain of An. coluzzii varied substantially. The used LLINs created a higher knock-down than mortality.

Conclusion
This study showed a high sporozoite infection rate and insecticide resistance towards pyrethroids and DDT in local malaria vectors. This strongly suggests that the risk of malaria transmission in Mweso and Kashuga is high and current malaria prevention methods in the population are only partially effective. PermaNet 3.0 might be considered as a new LLIN, as the side with PBO caused a high knock-down and mortality. Other nets may still provide physical protection. For efficient malaria vector control different types of insecticide should be chosen by MSF. As the only option for LLINs is a member of the pyrethroid family a member of another family (carbamate or organophosphate) should be chosen for IRS.
5. Introduction

5.1 Malaria in the Democratic Republic of Congo
Malaria is a major public health problem in the Democratic Republic of Congo (DRC) and therefore places the country as one of the most highly malaria-endemic countries in Africa [1]. The prevalence of malaria has been consistently reported high in various parts of the country [2]. The malaria burden is more compounded by the precarious conflict situations the country has experienced over the decades, especially in the eastern part of the country [2, 4].

5.2 Vector control program
Malaria transmission is characterised by different factors including the presence and abundance of efficient vectors, a suitable environment (temperature and humidity), a virulent parasite, and a susceptible human population. The malaria vectors in DRC include the mosquito species *Anopheles gambiae* sensu lato (s.l.), *An. funestus* s.l., *An. nili* and *An. moucheti* [5]. The distribution of these vectors is influenced by the prevailing environment. The implementation of a successful vector control program requires information such as the composition and abundance of the vector species in the area, the infectivity rate of these mosquitoes and the susceptibility of these vector mosquitoes to insecticides [6]. Due to the conflict situation in DRC, only limited information is available. Therefore entomological surveys are important before implementing vector control interventions.

5.3 Insecticide resistance
One of the most effective measures to control malaria is the use of Long Lasting Insecticide-treated Nets (LLINs). A LLIN is not only a physical barrier against vector mosquitoes, but the insecticides present on the bed net also repel and kill the mosquitoes. The only class of insecticides currently licenced by the World Health Organization (WHO) to impregnate bed nets are pyrethroids. This class of insecticides shows a low toxicity for mammals and a fast knock-down response in mosquitoes. Moreover, compared to other insecticide families, pyrethroids also have an excito-repellent effect, which can lead to a lower number of mosquitoes entering the house or disrupted blood feeding and premature exit of the mosquitoes from the house with the LLIN [7]. Pyrethroids act on the nervous system of the mosquito by changing the normal functioning of the *para*-type sodium channel [8]. It causes a prolonged opening of this channel, increasing the transmission of nerve pulses, which causes paralysis and eventually death of the mosquito. Recent vector control measures against malaria are hampered by the development of resistance against insecticides in vector populations [9]. Therefore a new generation of LLINs was developed to overcome insecticide resistance. These LLINs not only contain a pyrethroid, but also the synergist piperonyl butoxide (PBO) that reduces the level of (metabolic) insecticide resistance as it inhibits with enzymatic break-down processes of the insecticide in the mosquito. In this way, the PBO LLINs should have an increased killing effect on resistant malaria vectors, however, the evidence is still limited and WHO cannot yet justify a complete switch from pyrethroid-only LLINs to PBO LLINs in all kind of areas [10, 11]. All PBO LLINs have the interim recommendation status of WHO and they are now undergoing a WHOPES phase III evaluation to decide if they will get the full recommendation [12].

5
Resistance to insecticides encompasses physiological, biochemical, molecular and behavioural mechanisms [13, 14]. One important resistance mechanism against dichloro-diphenyl-trichloroethane (DDT) and pyrethroids is the knock-down resistance (kdr) mutation. Kdr is caused by a point mutation in the sodium channel and consists of two different forms [15]. One is called kdr-west and is predominantly found in West African countries. The mutation results in an amino acid change whereby leucine is replaced by phenyalanine (L1014F). The other form is called kdr-east and is most common in East African countries. Here leucine is replaced by serine (L1014S). Recently, heterozygous mosquitoes that carry both kdr-w and kdr-e mutations have been found as well [15].

5.4 Study Rationale
Médecins Sans Frontières-Operational Centre Amsterdam (MSF-OCA) operates in two areas in DRC: North Kivu and South Kivu. MSF is present in hospitals and health centres. Despite the various emergency health interventions (LLIN distribution, IRS, prompt and effective treatment) rolled-out by MSF-OCA in its operational areas, malaria transmission remains a public health challenge (data collected in hospitals and health centres supported by MSF). As part of the MSF-OCA strategic plan to fight malaria in its area of operations, a series of malaria research surveys were initiated to identify key malaria transmission indicators and plan appropriate interventions. The baseline malaria entomological survey reported here is part of a larger plan to answer key questions in relation to appropriate malaria control interventions.

6. Objectives

- To identify the main vector responsible for malaria transmission in the study area;
- To identify the sibling species of the main malaria mosquito vector(s) in the study area through molecular typing;
- To estimate the *Plasmodium falciparum* sporozoite infection rate in the collected vectors;
- To determine the level of susceptibility of *Anopheles gambiae* to insecticides;
- To determine the prevalence of knock-down resistance (*kdr*) mutation genes in *Anopheles gambiae* s.l.;
- To determine the effectiveness of new LLINs in the study area;
- To determine the residual insecticide activity of LLINs distributed in the study area;
7. Methods

7.1 Study area

Mweso and Kashuga are two villages located in the Masisi territory in the province North Kivu in the east of DRC (Figure 1). In Mweso a hospital and health center are supported by MSF and in Kashuga a health center and several health posts are supported. The villages are approximately 9km away from each other. Kashuga is characterized by the presence of three IDP camps. Mweso is approximately 1km$^2$ and Kashuga 2km$^2$ in size. In both villages agricultural fields are present and different types of insecticides are used on these fields for crop protection. In Table 2 the insecticides used by the agricultural institute in Mweso are shown and also all insecticides available on the market in Mweso are shown. North Kivu has a sub-tropical climate with annual temperatures ranging from 15 to 23°C. Rainfall is abundant in the rainy seasons from mid-August to mid-January and mid-February to mid-July with short relatively dry seasons from January to February and July to August every year.

![Map of Mweso and Kashuga](image)

Figure 1. The location of Mweso and Kashuga in the Democratic Republic of Congo (DRC).

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>Agricultural Institute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crop protection: Titan M 45 and Dichlorvos</td>
</tr>
<tr>
<td></td>
<td>Livestock protection: Deltox, Cypermethrin 5 and 30%, Vectocide, Megatix and Norotraz</td>
</tr>
<tr>
<td>Market</td>
<td>Dichlorvos, Alamycin Aerosol, Safari zeb (Mancozeb 80%), Cyper laser, Baygon and Tox BOP insecticide</td>
</tr>
</tbody>
</table>

In Mweso and Kashuga, MSF-OCA has been distributing LLINs in a targeted manner (via ANC programs and to malaria patients <5 years old at OPDs). The health plan of the Ministry of Health (MoH) includes a ‘blanket’ LLIN distribution every two years, the latest was in 2016. If security allows Fendona ($\alpha$-cypermethrin) is used twice a year for IRS in the hospital and health centers supported by MSF-OCA. In the past all houses in Kashuga town including the houses in the IDP camps were also regularly sprayed with Fendona. Ficam (Bendiocarb) is ordered to be used in future IRS campaigns.
7.2 Study design and sampling procedure

In December 2016 we started with this vector study in North Kivu, because of security issues the study was interrupted and started again in January 2017. The number of samples collected in 2015 was limited and therefore it was decided to not include these data in this report.

CDC light traps were used to collect mosquitoes during night, to get an insight in the mosquito species present in the area. Only in Kashuga CDC light traps were installed. Ten CDC light traps were installed on average twice a week from 23rd of January – 26th of April 2017. Traps were installed inside 269 selected bed rooms after verbal consent had been sought from occupants of the rooms (Figure 2). Houses were randomly selected in all neighbourhoods of Kashuga. The houses were geo-referenced with a hand-held GPS device. The following characteristics were recorded for each house: type of roofing material, type of walls, presence of a net, use of mosquito preventive measures in the night prior to the survey, and number of occupants in the house the night before mosquito collection (See appendix 12.5). Traps were placed approximately 1.2m above the floor at the foot-end of the persons who sleep in the room and have consented to receive traps. Traps were switched on by community workers from about 6:00 pm and removed by 06:00 am on each trapping day. As part of ethical requirements, the participating persons slept under their own bed net or a bed net was provided by the team. The collected mosquitoes were identified to species level, and abdominal state (unfed, blood fed, half-gravid or fully gravid) was recorded. Mosquitoes were put into micro-centrifuge tubes of which the lids were pierced with a needle. A maximum of ten mosquitoes of the same species and abdominal state were placed in the same tube and placed into zip-lock plastic bags containing desiccants. The pierced tubes, but closed ziplock bag reduced moisture content inside the tube and prevented decay of the samples.

Figure 2. The 269 houses in Kashuga where CDC light traps were installed.
7.3 Long Lasting Insecticide-treated Nets (LLINs) collections
Thirty-two (32) LLINs were randomly collected from pregnant women visiting the maternity department in the health center of Kashuga (PermaNet 2.0=11; DuraNet=5; Netprotect=1; Olyset=2; Yorkool=13). The midwives were instructed to fill out a short questionnaire when a woman brought her old LLIN. The questions were about the age of the bed net, the usage and function of the net, how many times the net was washed and how it was dried (See appendix 12.5). A new LLIN was given to the pregnant woman when she brought her old one. From each net, three square panels (30cm x 30cm) were cut (roof, length and width sections). Samples of net panels were packed in aluminium foil containing desiccants, properly labelled and stored at room temperature until shipped to the Netherlands to determine their residual insecticide activity.

7.4 Laboratory analysis

7.4.1 Collection of larvae for insecticide exposure assays
An. gambiae larvae were collected from breeding sites in Kashuga and Mweso to create our own An. gambiae rearing. The larvae were grown to adult mosquitoes in separate cages so a difference could be made between mosquitoes from Kashuga and mosquitoes from Mweso. Several times a week new larvae were collected to have adult mosquitoes. In Kashuga the main breeding sites were four different fish ponds close to the Ibuga camp but also stagnant water pools in Kashuga were used. In Mweso larvae were collected in several stagnant water pools in agricultural fields. These mosquitoes were used to perform several different experiments: CDC bottle bioassays, WHO insecticide susceptibility tests and WHO cone bioassays.

7.4.2 CDC bottle bioassay
A CDC botte bioassay was performed with the insecticide α-cypermethrin (pyrethroid), because this insecticide is used by MSF for IRS. With the CDC bottle bioassay, different concentrations of the same insecticide can be tested and the level of resistance in the mosquito population can be determined. Because the diagnostic dose was not known it was chosen to test four different concentrations of α-cypermethrin in a wide range: 200 mg/L, 20 mg/L, 0.32mg/L and 0.01mg/L. The α-cypermethrin was dissolved in 95% ethanol. The experiment was performed according to the guidelines of the CDC [16]. For the experiment non-blood fed 3-5 day old female mosquitoes from our own Kashuga rearing were used. The experiment was performed at 27 ± 0.8°C and a relative humidity of 70 ± 7%.

The diagnostic dose for α-cypermethrin for An. gambiae was unknown, therefore the CDC bottle bioassay was also performed in the Laboratory of Entomology, Wageningen University (The Netherlands) with susceptible Anopheles coluzzii mosquitoes. Via this way the diagnostic time and diagnostic dose were set on 30 minutes at a concentration of 12.5 mg/L, because with this time and concentration 100% of the mosquitoes died.
7.4.3 Insecticide susceptibility test

Bioassays to test insecticide susceptibility were performed at the field laboratory in Mweso with discriminating dosages of seven insecticides: DDT 4% (organochlorine), bendiocarb 0.1% (carbamate), α-cypermethrin 0.05% (pyrethroid), permethrin 0.75% (pyrethroid), deltamethrin 0.05% (pyrethroid), malathion 5.0% (organophosphate) and pirimiphos-methyl 0.25% (organophosphate). All bioassays were performed with An. gambiae from Kashuga and Mweso, except for permethrin and DDT. These were only tested with mosquitoes from Kashuga. The bioassays were performed using WHO baseline susceptibility test kits and procedures were aligned with WHOPES guidelines [17]. Non-blood fed 3-6 day old female mosquitoes were randomly selected for the insecticide susceptibility test. The number of mosquitoes knocked-down was recorded during one hour. After exposing mosquitoes for one hour, they were held in WHO holding tubes and fed on sugar water via pieces of cotton wool on top of the tube. The overall mortality was recorded 24 hours after starting the test.

7.4.4 WHO Cone-bioassays (Mweso and Wageningen)

To test the efficacy of new LLINs on the local mosquitoes from Mweso and Kashuga, a standardized WHO cone-bioassay was performed in Mweso [19]. Non-blood fed 3-7 day old female An. gambiae were used. The pieces of 30x30 cm were attached to a wooden frame which was placed under a 45-degree angle. Five An. gambiae were released in one transparent plastic cone that was placed on the wooden frame. Five of these cones with mosquitoes were placed on a single piece of net simultaneously. The mosquitoes were exposed to the net for three minutes. After this, they were transferred to paper cups and had access to a sugar solution via a damp cotton wool. One hour after the start of the test, the number of mosquitoes knocked-down was recorded and 24 hours after the start of the test the mortality was recorded. In total 7 LLIN pieces were tested: 2x the roof panel of PermaNet 3.0 (PBO side), 2x the side panel of PermaNet 3.0 (non-PBO side), 2x the side panel of Olyset plus and a side panel of Netprotect. Bioassays were carried out at 26.5 ± 3.5°C and 68.0 ± 10.0% relative humidity.

To test the efficacy of used LLINs collected from Kashuga, a standardized WHO cone-bioassay was performed with non-resistant An. coluzzii in the Netherlands. In total, 17 LLINs were randomly selected: 5 nets each from the brands PermaNet 2.0 and Yorkool, 4 nets from the brand DuraNet, 2 nets from the brand Olyset and 1 net from the brand Netprotect. From all nets the width section was taken to expose mosquitoes in the cone. Five susceptible, non-blood fed, 4-6 day old female Anopheles coluzzii (SUAKOKO strain, Liberia) were released per cone. Every day different nets and one control net (untreated piece of net) were tested in random order. In total, 50 mosquitoes (two replicates, each replicate consisted of 5 cones with in total 25 mosquitoes) were exposed to each piece of net. Bioassays were carried out at 28.0 ± 2.5°C and 73.5 ± 17% relative humidity.

Definitions used for knock-down and mortality were recommended by WHOPES [20]. Mosquitoes that were moribund or dead were recorded as knocked-down one hour post-test and as dead 24 hours post-test. A mosquito was moribund if it could not stand or fly in a coordinated way or took off briefly but fell immediately. A mosquito was considered dead if it was immobile, could not stand or showed no sign of life. It was considered alive otherwise.
7.4.5 Mosquito DNA extraction
After shipment of the mosquito samples to the Laboratory of Entomology of Wageningen University, The Netherlands, DNA was extracted from these mosquitoes. Collected mosquitoes were grinded as described in the protocol (Appendix 12.1). DNA extracts were used for Polymerase Chain Reaction (PCR) to determine sibling species (7.4.6), the presence of the kdr mutation (7.4.7) and for ELISA (7.4.8).

7.4.6 Sibling species determination by Polymerase Chain Reaction (PCR)
During this study only mosquitoes from the An. gambiae complex were collected. This complex consists of sibling species that are morphologically similar and can only be distinguished by using molecular techniques. As a result of their different preferences for human and/or animal hosts, the vectorial capacities of these sibling species differ. It is therefore important to determine the sibling species level of the collected mosquitoes [18]. During this study 324 An. gambiae s.l. were collected and tested, the protocol is described in appendix 12.3.

7.4.7 Detection of the knock-down resistance (kdr) mutation
All An. gambiae s.l. were tested by PCR to detect kdr mutations. Kdr is a point mutation which gives an indication of resistance against DDT and pyrethroids. We tested for both types of kdr mutations (kdr-east and kdr-west). The protocol used for this PCR is described in appendix 12.4. Next to the An. gambiae s.l. collected by CDC light traps, also the An. gambiae sensu stricto (s.s.) used in the insecticide susceptibility assay were tested for the presence of kdr-mutations. The behaviour and susceptibility of these mosquitoes towards pyrethroids and DDT is known, and in this way the relation between resistance phenotype and a resistance genotype could be determined.

7.4.8 Circumsporozoite (CS) ELISA
To test whether the mosquitoes were carrying Plasmodium falciparum sporozoites, a CS-ELISA was performed. The protocol is described in appendix 12.2.

7.5 Data analysis and interpretation
The Sporozoite Rate (SR) is calculated as the proportion of Anopheles mosquitoes tested positive by CS ELISA. Entomological Inoculation Rates (EIR) were calculated for different areas. The EIR is the product of the sporozoite rate and the average number of Anopheles mosquitoes per house per person collected by CDC light traps in the area. Latter number is obtained by dividing the number of mosquitoes caught in the house by the average number of people sleeping in the house. The product is multiplied by 365 days to get an estimate for EIR expressed as the number of infective bites per person per year. The EIR gives an indication about the risk of people being bitten by a malaria infected mosquito at the time of sampling.

Replicate insecticide susceptibility test results, CDC bottle bioassay test results and WHO cone bioassay results were pooled and analysed. Percentages from susceptibility tests were compared to World Health Organisation Pesticides Evaluation Scheme (WHOPES) recommended ranges. Mosquito populations with a mortality >98% are considered susceptible, between 90-98% indicates resistance but requires confirmation of resistant genes in the population and mortality less than 90% confirms resistance [17]. Mortalities were corrected by Abbott’s formula when the mortality ranged between 5-10% in the control experiments.
8. Results

8.1 Mosquito densities and species composition

In total, 1,692 mosquitoes were collected with CDC light traps in bed rooms of 269 different houses. The majority of these mosquitoes were *Culex* spp. (80.7%) followed by the malaria vector *An. gambiae* s.l. (19.2%) (Table 2). *An. gambiae* s.l. was the only malaria vector found in Kashuga. The majority of these mosquitoes was unfed and thus host-seeking (96.9%), a small proportion was blood fed (3.1%), indicating that these mosquitoes had taken a blood meal successfully recently. No half gravid or gravid mosquitoes were collected. On average, two anophelines per bed room per night were collected with a maximum of 20 anophelines in one bed room during one night.

Table 2. Numbers of mosquitoes collected with CDC light traps (n=269) in Kashuga.

<table>
<thead>
<tr>
<th>Species</th>
<th>Unfed</th>
<th>Blood fed</th>
<th>Total</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em> s.l.</td>
<td>314</td>
<td>10</td>
<td>324</td>
<td>19.15</td>
</tr>
<tr>
<td><em>Culex</em> spp.</td>
<td>1353</td>
<td>13</td>
<td>1366</td>
<td>80.73</td>
</tr>
<tr>
<td><em>Mansonia</em> spp.</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0.12</td>
</tr>
<tr>
<td>Total</td>
<td>1669</td>
<td>23</td>
<td>1692</td>
<td></td>
</tr>
</tbody>
</table>

With a species PCR the sibling species of the *An. gambiae* s.l. complex were determined. Results are shown in Table 3. The majority of the mosquitoes were *An. gambiae* s.s., only five *An. arabiensis* were found.

Table 3. Sub-species determination of *An. gambiae* s.l.

<table>
<thead>
<tr>
<th>Species</th>
<th>Subspecies</th>
<th>Number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em> s.l.</td>
<td><em>An. gambiae</em> s.s.</td>
<td>299</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td><em>An. arabiensis</em></td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>no signal</td>
<td>20</td>
<td>6.2</td>
</tr>
</tbody>
</table>

8.2 Data collected at the households

A questionnaire was taken at the 269 households where CDC light traps were installed and the houses were inspected by the team. A total of 75.8% of the households had at least 1 LLIN, in Table 4 the number of LLINs per household is shown. On average 6 people are living in one house and they own on average 1 LLINs for the whole household. Universal coverage is defined as the use of LLINs by all household members regardless of age or gender, typically implemented as at least 1 LLINs for every two household members [21]. According to this definition Kashuga reaches a universal coverage of only 39.6%.
Table 4. Number of LLINs found per household.

<table>
<thead>
<tr>
<th>Number of LLINs</th>
<th>Number of households</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65</td>
<td>24.2</td>
</tr>
<tr>
<td>1</td>
<td>119</td>
<td>44.2</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>24.9</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Of the participating households 75.8% (204/269 households) were displaced. On average, these people reported to have already been displaced for 2.7 years. Therefore the quality of the houses was not good and 91.1% of the houses in this study had eaves, thereby facilitating mosquitoes to enter the house.

In Kashuga none of the households had electricity. On average people enter their house around 7:00 pm and go to sleep around 8:30 pm, people wake up early morning just before 6:00 am.

8.3 *P. falciparum* sporozoite rates

All *An. gambiae* s.l. (324) were screened by ELISA to determine whether the mosquitoes were positive for *P. falciparum* sporozoites. In total, 45 mosquitoes were positive, a maximum of five positive mosquitoes per house was found (Figure 3). The overall Sporozoite Rate (SR) of anophelines was thus 13.9% (45/324). The majority of infected mosquitoes was found in the area called Ibuga, an IDP camp on the hills in the north of Kashuga. Quite strikingly, in the north part of the Ibuga camp 18.6% of all houses (52/269) carried two third (66.7%; 30/45) of all infected mosquitoes.

![Image of mosquito distribution map](image-url)

*Figure 3. The number of *P. falciparum* positive mosquitoes per house in Kashuga.*
To show the heterogeneity of malaria transmission, the Entomological Inoculation Rate was calculated for Kashuga (269 houses) and also for the north of the Ibuga camp alone (52 houses) and Kashuga minus the north of the Ibuga camp (217 houses). The EIR is defined as the number of infective bites received by an individual per unit time, in this study we calculated it per year. The EIR for the entire Kashuga area is 10.5 infective bites per person per year, based on 1.2 mosquitoes per night, a sporozoite rate of 13.9% and 6 persons per house. For the north of the Ibuga camp the EIR is 40.5 infective bites per person per year, based on 4.3 mosquitoes per night, a sporozoite rate of 13.5% and 5.2 persons per house. The EIR for Kashuga minus the north of the Ibuga camp is 4.3 infective bites per person per year, based on 0.47 mosquitoes per night, a sporozoite rate of 14.7% and 5.9 persons per house.

8.4 CDC bottle bioassay
The mortality of local *An. gambiae* after exposure to four different concentrations of α-cypermethrin is shown in Figure 4. After two hours 100% of the mosquitoes died at a concentration of 200 mg/L, 86.0% of the mosquitoes died at 20 mg/L, 10.0% died at 0.32 mg/L and 0% at a concentration of 0.01 mg/L. This same experiment was done at the Laboratory of Entomology in the Netherlands with susceptible *An. coluzzii* to determine the diagnostic time and diagnostic dose. The diagnostic time is 45 min with a diagnostic dose of 0.32 mg/L. The local mosquitoes of Kashuga showed a mortality of only 8.5% after 45 minutes at a concentration of 0.32 mg/L, and are therefore highly resistant to α-cypermethrin.

![Figure 4. Mortality of local An. gambiae exposed to four different concentrations of α-cypermethrin. The black dotted line shows the diagnostic time when the diagnostic dose is 12.5 mg/L. Error bars show standard error of the mean.](image-url)
8.5 Insecticide susceptibility test

The knock-down effect of several different insecticides was determined for *An. gambiae* s.l. (mainly *An. gambiae* s.s.) collected from Kashuga and Mweso. Malathion showed a high knock-down effect for both locations, after 1 hour 100% of the *An. gambiae* s.l. were knocked-down. Also bendiocarb had a high knock-down effect, 100% and 94.1% for respectively Kashuga and Mweso. The knock-down effect of the other insecticides was much lower. Pirimiphos-methyl had a knock-down of 64.4% (Kashuga) and 11.2% (Mweso), deltamethrin 53.4% (Kashuga) and 41.6% (Mweso), alphacypermethrin 51.3% (Kashuga) and 19.8% (Mweso). DDT and permethrin were only tested with mosquitoes from Kashuga and had both a very low knock-down effect. After 1 hour 9.6% of the mosquitoes were knocked-down after exposure to DDT and only 1.7% was knocked-down after exposure to permethrin. All results are shown in Figure 5A and B.

![Figure 5A](image1.png)  
*Figure 5A. Knock-down of *An. gambiae* s.l. from Kashuga to seven insecticides. Error bars show standard error of the mean.*

![Figure 5B](image2.png)  
*Figure 5B. Knock-down of *An. gambiae* s.l. from Mweso to five insecticides. Error bars show standard error of the mean.*
Twenty-four hours after exposure, the mortality for mosquitoes exposed to malathion was 100%. Bendiocarb caused a mortality of 98.8 (Kashuga) and 96.8 (Mweso), pirimiphos-methyl 100% (Kashuga) and 72.3% (Mweso), deltamethrin 65.7% (Kashuga) and 54.9% (Mweso), α-cypermethrin 50.0% (Kashuga) and 34.4% (Mweso), DDT 21.2% (Kashuga) and permethrin 9.2% (Kashuga). All results are shown in Figure 6. Mortality below 90% indicates resistance development in the mosquito population. Clear resistance is therefore shown for DDT and all pyrethroids tested.

Figure 6. Mortality (24 hour post exposure) of An. gambiae s.l. from Kashuga and Mweso. A mortality below 90% indicates resistance (indicated by the horizontal black dashed line). Error bars show standard error of the mean.
8.6 Knock-down resistance

8.6.1 Knock-down resistance mutation in collected mosquitoes

With a PCR test 304 An. gambiae s.l. were screened for the presence of the knock-down resistance (kdr) mutation. For both kdr-east and kdr-west was tested. The heterozygote resistant or homozygote resistant genotypes indicate the presence of genetic based resistance towards DDT and pyrethroids. 2.8% of the mosquitoes showed the presence of both resistance genotype types in one individual. Only four (1.2%) mosquitoes showed the susceptible genotype for both kdr mutations, three of them were An. arabiensis. The Kdr-e resistant mutation was present in 89.5% of the mosquitoes, while the resistance mutation of kdr-w was present in only 21.4% of the mosquitoes (Table 5).

Table 5. Knock-down resistance genotype of collected An. gambiae s.s. (n=299) and An. arabiensis (n=5).

<table>
<thead>
<tr>
<th></th>
<th>Kdr-east</th>
<th></th>
<th></th>
<th>Kdr-west</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>An. gambiae s.s.</td>
<td>%</td>
<td>An. arabiensis</td>
<td>%</td>
<td>An. gambiae s.s.</td>
<td>%</td>
</tr>
<tr>
<td>Homozygote resistant</td>
<td>178</td>
<td>59.5</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>92</td>
<td>30.8</td>
<td>2</td>
<td>40</td>
<td>52</td>
<td>17.4</td>
</tr>
<tr>
<td>Homozygote susceptible</td>
<td>1</td>
<td>0.3</td>
<td>3</td>
<td>60</td>
<td>172</td>
<td>57.5</td>
</tr>
<tr>
<td>No clear signal</td>
<td>28</td>
<td>9.4</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>20.7</td>
</tr>
</tbody>
</table>

8.5.2. Knock-down resistance mutations in mosquitoes from the susceptibility assay

The mosquitoes, collected in the field as larvae and exposed as adults to DDT, α-cypermethrin, deltamethrin and permethrin in the insecticide susceptibility assay were screened for the presence of knock-down resistance mutations. This was done to determine the relation between the knock-down resistance genotype and the phenotype (mortality) of the mosquitoes. When a mosquito showed the presence of a heterozygote or homozygote resistance genotype, it is expected that this individual would remain alive after exposure to the insecticides, a homozygote susceptible individual is expected to die. In total, 49.6% of the mosquitoes showed the expected relationship, 50.4% of the mosquitoes died while they had the heterozygote or homozygote resistant genotype. According to our expectations, none of the mosquitoes that had the susceptible genotype stayed alive after exposure to the insecticides (Table 6). Interestingly, the one mosquito with a susceptible genotype for both kdr-east and kdr-west was the only An. arabiensis tested.

Table 6. Knock-down resistance genotype of the mosquitoes used in the insecticide susceptibility bioassay. In green the combination that was expected in red the unexpected result.

<table>
<thead>
<tr>
<th></th>
<th>Alive (+)</th>
<th>Dead (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant/Heterozygote (+)</td>
<td>66</td>
<td>68</td>
<td>134</td>
</tr>
<tr>
<td>Susceptible (-)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>69</td>
<td>135</td>
</tr>
</tbody>
</table>
8.7 WHO cone bioassays Mweso and Wageningen

In Mweso, a total of 7 new LLIN pieces were tested with mosquitoes from Kashuga and Mweso (PermaNet 3.0 PBO side=2, PermaNet 3.0 non-PBO side=2, Olyset Plus=2, Netprotect=1). The results of this experiment are shown in Figure 7. Table 7 shows the details of the LLINs tested in Mweso. A clear difference is seen in the knock-down and mortality created by the roof part of the PermaNet 3.0 compared to the side panel. Also the Olyset plus net is causing a higher knock-down and mortality compared to the net pieces without PBO, however the effect is much less than with the roof panel of the PermaNet 3.0.

![Figure 7. The knock-down (blue) and mortality (red) of local mosquitoes exposed to different brands of LLINs during the WHO cone bioassay in Mweso.](image)

**Table 7. Active compounds and materials of LLINs tested in Mweso.**

<table>
<thead>
<tr>
<th></th>
<th>PermaNet 3.0 Roof panel</th>
<th>PermaNet 3.0 Side panel</th>
<th>Olyset Plus All panels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active compounds</strong></td>
<td>Deltamethrin (4.0 g/kg) + PBO (25 g/kg)</td>
<td>Deltamethrin (2.8 g/kg)</td>
<td>Permethrin (20 g/kg) + PBO (10 g/kg)</td>
</tr>
<tr>
<td><strong>Material</strong></td>
<td>polyethylene</td>
<td>polyester</td>
<td>polyethylene</td>
</tr>
</tbody>
</table>
In Wageningen used LLINs collected in Kashuga were tested with non-resistant *An. coluzzii*. The insecticidal residual activity of the used LLINs varied substantially not only among the different brands but also within the same brand. Per net sample 50 mosquitoes were exposed for 3 minutes. The knock-down (1 hour after the start of the test) and mortality (24 hours after the start of the test) are shown in Figure 8 and Figure 9, respectively. The knock-down effect, ranging from 88 to 100%, created by the nets was much higher than the mortality. The two Olyset nets caused the lowest mortality, 22 and 36% respectively.

*Figure 8. Percentage of susceptible An. coluzzii knocked-down per net per brand (n=50 mosquitoes per net sample).*

*Figure 9. The percentage of dead susceptible An. coluzzii per net per brand (n=50 mosquitoes per net sample).*
Table 8. Active compounds and materials of LLINs tested in Wageningen.

<table>
<thead>
<tr>
<th></th>
<th>DuraNet</th>
<th>Netprotect</th>
<th>Olyset</th>
<th>PermaNet 2.0</th>
<th>Yorkool</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active compounds</strong></td>
<td>α-cypermethrin 5.8g/kg</td>
<td>deltamethrin 1.8g/kg</td>
<td>permethrin 20g/kg</td>
<td>deltamethrin 1.4-1.8g/kg</td>
<td>deltamethrin 55 mg/m²</td>
</tr>
<tr>
<td><strong>Material</strong></td>
<td>polyethylene</td>
<td>polyethylene</td>
<td>polyethylene</td>
<td>polyester</td>
<td>polyester</td>
</tr>
</tbody>
</table>
9. Discussion

During this study the main malaria vector collected was *An. gambiae* s.l. of which the majority was *An. gambiae* s.s. No *An. funestus* were collected during this study, probably due to a lack of suitable breeding sites. A typical *Anopheles funestus* breeding site is a large, permanent or semi-permanent body of fresh water especially with vegetation. *An. gambiae* s.s. is highly anthropophilic and endophilic, and is therefore an excellent vector for human malaria. Similar to the vector studies performed in Shamwana (2013) and Baraka (2015), but in contrast to many other areas in Africa, only a few *An. arabiensis* were found [22]. This might be explained by the lower selection pressure on the indoor feeding *An. gambiae* s.s. due to a lack of vector control tools available in the area [23]. This vector study and a Knowledge, Attitudes and Practice survey (KAP) performed by MSF in 2013 showed that Mweso and Kashuga perform poor on the coverage and usage of LLINs [24].

The mosquitoes collected during this study showed a *Plasmodium falciparum* sporozoite rate of 13.9%. This is much higher than the mean sporozoite rate of 5.1% found in *An. gambiae* s.l. by the President’s Malaria Initiative (PMI) in seven sentinel sites (Kingasani, Kalemie, Katana, Mikalayi, Lodja, Kabondo and Kapolowe) in different provinces in DRC [25]. In our study, we may have overestimated the number of positive mosquitoes, because of an extremely low variation in the negative control samples of the ELISA. This resulted in a low cut-off value and therefore a higher number of mosquitoes assigned as ‘positive’. Different cut-off values were calculated which resulted in a sporozoite rate ranging from 13.0% to 22.2%. We chose to go for the cut-off value that resulted in 13.9%, because this might already be an overestimation of the number of positive mosquitoes. The majority of infected mosquitoes was found in the Ibuga camp, in the north of the study area. At the border of this camp there are several fish ponds located that are ideal breeding sites for *An. gambiae* s.l.. This was confirmed as we collected many larvae there. The people living in Ibuga camp have houses of poor quality. The majority of the houses has eaves, thereby facilitating mosquitoes to enter the house. Moreover, 34.3% of the households in the Ibuga camp did not have any LLIN to protect them and in the other households universal coverage (1 LLINs per 2 persons) was not reached. Earlier work demonstrated that the flight distance of *An. gambiae* s.l. ranged between a mean distance of 350-650m a day and a maximum distance of 3.6km [26, 27, 28]. This means that mosquitoes emerging from the fish ponds at the border of the Ibuga camp can reach the entire camp and some of them may even reach the city center of Kashuga. Besides that, the IDPs living in the camps in Kashuga often flee away from higher areas and have thus rarely been exposed to malaria before. This makes these people vulnerable for malaria, especially the ones living in the Ibuga camp.

The calculations of the Entomological Inoculation Rate (EIR) showed us that malaria transmission is highly heterogeneous in the study area varying from approximately 4 infective bites per person per year in the area of Kashuga minus the north of the Ibuga camp to approximately 40 infective bites per person per year in the north of the Ibuga camp. This shows again that extra attention needs to be paid to the northern part of the Ibuga camp as malaria transmission is much higher there.
Previously, in Wageningen a CDC bottle bioassay was performed to determine the diagnostic time and dose of α-cypermethrin. The diagnostic time was set at 30 minutes with a concentration of 12.5 mg/L α-cypermethrin. This concentration was not tested in the field but we can conclude that at a concentration of 12.5 mg/L α-cypermethrin the mortality will be less than 22% as this was the mortality at 30 minutes with a concentration of 20 mg/L α-cypermethrin. This confirms high resistance towards α-cypermethrin in the local mosquito population. This might be explained by repeated exposure of mosquitoes in the study area to this insecticide via LLINs (DuraNet) and IRS (Fendona).

Local mosquitoes from Kashuga and Mweso were exposed to seven different insecticides during a WHO tube bioassay. Bendiocarb and malathion caused a clear knock-down and mortality. Therefore, both insecticides can be considered as candidate insecticides for vector control activities. Pirimiphos-methyl caused a lower knock-down, but after 24h 100% of the mosquitoes from Kashuga had died, so this insecticide can also be considered as a candidate insecticide. Quite strikingly, a different result was seen for the mosquitoes from Mweso (collected only approximately 9km away from Kashuga) that were exposed to pirimiphos-methyl. Of these, only 72% died after 24h. The experiment with pirimiphos-methyl consisted of five replicates of which three were performed simultaneously on the same day. These three replicates gave a much lower mortality than the other two replicates, which caused a mortality of 100%. Maybe this was due to very local resistance to pirimiphos-methyl, found in the one batch of mosquitoes used during that day. Another explanation could be that the insecticide treated papers were not well impregnated. However, this is unlikely because all insecticide treated papers come from the WHO reference center in Malaysia. Another technical error for instance bad closing of the storage box where the insecticide treated papers were kept, or an environmental condition that reduces the toxicity of the insecticide could also be a reason for the resistance found in this one batch of mosquitoes, therefore more investigation is needed. The knock-down effect and mortality of the pyrethroids α-cypermethrin, deltamethrin and permethrin and the organochlorine DDT was much lower and suggests resistance towards these insecticides. The development of resistance towards pyrethroids is worrying because this is the only insecticide family licenced to be used on LLINs. There are limited malaria vector control tools in the area therefore the resistance towards pyrethroids might be explained by the use of pesticides in agriculture. In Mweso there is an agricultural institute where they are using pyrethroids without any clear schedule. These pesticides are also available for everyone on the market and even in the MSF compound pyrethroid insecticides are used for pest control in the garden.

The presence of kdr mutations in the local vector mosquitoes supports the development of resistance towards DDT and pyrethroids in An. gambiae s.l.. The PCR of the knock-down resistance mutation showed that both types of mutations, kdr-west and kdr-east, are present in Kashuga. Three out of the four individuals with a susceptible genotype were An. arabiensis. This is in line with other studies showing that kdr mutations are predominantly found in An. gambiae s.s., probably due to differences in insecticide pressure selection because An. gambiae s.s. is more endophilic [29, 30]. By also screening the adult mosquitoes collected as larvae used in the WHO insecticide susceptibility bioassay for kdr mutations, the relation between a resistant phenotype and genotype could be investigated. In line with our expectations, none of the mosquitoes that stayed alive after exposure to a pyrethroid or DDT carried a susceptible genotype for both kdr-east and kdr-west. In contrast, there were mosquitoes that died
after exposure to the insecticide while they were carrying a homozygote or heterozygote \textit{kdr} mutation, although this is less of an operational concern. This might be explained because the \textit{kdr} genotype can only explain a portion of the heritable variation in resistance \cite{31,32}. Also in this experiment the one mosquito carrying both susceptible genotypes was the only \textit{An. arabiensis} tested.

The efficacy of used LLINs collected from pregnant women in Kashuga varied substantially. The knock-down effect was higher compared to the mortality after 24h. Especially the used Olyset caused a much higher knock-down than mortality. It seems that there is still some insecticide left on the LLINs but not enough to kill susceptible mosquitoes. Nets may have lost their insecticides due to a variety of factors. Majority of the people said to wash their LLIN on a regular basis and dry them in direct sun light, thereby reducing the insecticides present on the net. Therefore the LLIN now only represent a physical barrier that limits mosquito biting during the night. Moreover, universal coverage of LLINs was not reached in Kashuga, this might be because MoH is not doing a proper mass distribution of LLINs.

The new generation LLINs, PermaNet 3.0 and Olyset Plus, were tested with local mosquitoes during a WHO cone bioassay. PermaNet 3.0 is a LLIN made of two different fabrics. The side panels of the net are of polyester and coated with deltamethrin and the roof is made of polyethylene which is incorporated with the synergist piperonyl butoxide (PBO) and deltamethrin. The deltamethrin concentration is higher on the roof compared to the side panels. Olyset Plus is made of one material, polyethylene incorporated with the synergist PBO and permethrin. For PermaNet 3.0 a side panel and roof panel were tested to see the difference with and without PBO. A clear difference was seen between these panels. The non-PBO side had no effect on the mosquitoes while the PBO side killed almost 100\% of the mosquitoes. This may be due to the combination of a higher concentration of deltamethrin and the synergistic effect of PBO, but these two effects could not be separated in this study. A different effect was seen for the Olyset Plus net. The overall knock-down and mortality induced by the net was much lower than for PermaNet 3.0. This might be explained by a lower dosage of PBO. The roof panel of PermaNet 3.0 is incorporated with 25 g/kg PBO and the entire Olyset Plus net with 10 g/kg PBO. Besides that, the local mosquitoes seemed to be more resistant towards permethrin than towards deltamethrin. However, the LLINs were only tested with a WHO cone bioassay and no clear conclusion can be drawn on how the net will perform when it is hanging inside a hut. Other studies with an experimental hut set-up have shown that a PBO net is working more efficiently with pyrethroid resistant mosquitoes than a normal LLINs and untreated nets \cite{33,34}. The PBO nets still show a higher knock-down and mortality than the Netprotect without PBO so it is advised to start using PBO nets in the area. The fact that more mosquitoes die when they are exposed to PBO suggests that metabolic resistance also plays a role in the mosquitoes, because PBO blocks the enzymatic break-down of insecticide molecules, thereby enhancing the effect of the insecticide.

Several new vector control tools to fight insecticide resistance are now under development, such as the mixture LLIN, Interceptor® G2, with the pyrethroid \textit{α}-cypermethrin combined with the insecticide chlorfenapyr, a member of the pyrrole family \cite{35}. Chlorfenapyr has a different mode of action than the pyrethroids. It is not neurotoxic, but causes disruption of cellular respiration and oxidative phosphorylation in mitochondria. Therefore, it is still effective against pyrethroid resistant mosquitoes.
Chlorfenapyr lacks the excito-repellency effect, but by combining it with a pyrethroid in the same net this problem is solved. The study of Ngufor et al. (2017) looked into the efficacy of theInterceptor® G2, compared to a combination of chlorfenapyr IRS and a standard \( \alpha \)-cypermethrin LLINs and a pyrethroid-only net alone or chlorfenapyr IRS alone in an experimental hut set up in Benin [36]. Both the mixture LLIN and the combination of a pyrethroid-only net and chlorfenapyr IRS showed to be more efficient resulting in higher mortality than a pyrethroid-only net or chlorfenapyr IRS alone. It is also recommended for Kashuga and Mweso to use a combination of different insecticide families, pyrethroids with PBO for LLINs and carbamates and organophosphates for IRS to control malaria vectors in the area. Since rotation of the insecticides is important to slow down the development of resistance, it will be important to change the family of insecticides used for IRS (carbamate and organophosphates), because changing the insecticide on LLINs is not yet possible.
10. Conclusions and recommendations

At the time of this study the dominant malaria vector was *An. gambiae* s.l., an excellent vector of human malaria. The majority of *Plasmodium falciparum* infected mosquitoes was found in the Ibuga camp, on the hills of Kashuga, north of the study area. Fish ponds on the border of the camp showed to be the main breeding site of the mosquitoes. The sporozoite infection rate found in Kashuga was very high compared to other areas, leading to a high risk of transmission, even when there are not so many mosquitoes around. The EIR showed that malaria transmission is highly heterogeneous in Kashuga, differing from 4 infective bites per person per year in Kashuga minus the north part of the Ibuga camp to 40 infective bites per person per year in the north of the Ibuga camp. With the fish ponds close to the Ibuga camp creating an ideal breeding site for *An. gambiae* s.l., it is advised to look into the opportunities of larval control and breeding site destruction, thereby reducing the number of adult mosquitoes. Another recommendation will be to change the insecticide used for IRS into a member of the carbamates or the organophosphates. Our study showed that a pyrethroid is not effective in killing anophelines. Furthermore, it is not recommended by WHO to use pyrethroids for IRS because this insecticide family is the only one that is licensed to be used on LLINs. Rotation of this insecticide will be important to slow down resistance development. If security does not allow to spray regularly, a possibility is to focus on the distribution of LLINs with an intensive sensitization campaign of the local population. Explaining them how, why and when to use a LLIN, and that one can still sleep under a LLIN that has a limited number of holes because holes can be repaired easily with a minimum of materials and skills. PBO LLINs might be a better option than regular LLINs, due to the insecticide resistance present in the local vector population.

This study showed that the risk of malaria transmission in Kashuga and Mweso is high and current malaria prevention methods are only partially effective. Different vector control tools should be used to control the local mosquito population.
11. References

5. WHO, DRC country profile malaria, July 2017
12. WHO, WHO recommended long-lasting insecticidal nets. (June 2017)


12. Annexes

12.1 Protocol DNA extraction

Unfed mosquitoes
(This protocol was modified after a protocol used by the Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen)

1. Individual mosquitoes should be placed into 1.5mL Eppendorf tubes and numbered.
2. The closed Eppendorf tube containing the mosquito is held into liquid nitrogen for a few seconds, to quickly freeze the mosquito and facilitate grinding.
3. Grinding should be carried out using 1.5mL tube specific pestles\(^a\) and should be done using an automatic pestle grinder\(^b\). Grind until no large body parts are visible (5-10 seconds). It can help to carefully hold the bottom of the tube in the liquid nitrogen to freeze the mosquito parts again.
4. Add 250 µL of grinding buffer containing 1xPBS (pH 7.4) with 1% Sarcosil\(^c\) and 0.05% Tween 20. When the sample is grinded, ensure that the pestle is spun out of the sample (but within the tube) to minimise sample loss. Some loss is however inevitable. Ensure that as little liquid and body parts remain stuck to the pestle.
5. Close the Eppendorf tube, pulse in the micro-centrifuge, and move to storage at -20°C or -80°C.
6. After sample grinding, pestles should be moved to a glass container with water/bleach (approx. 5%). Pestles can be rinsed with water several times and drained. Pestles should then be dried with paper towels, and prepared for autoclaving. For autoclaving pestles can be grouped in glass beakers and these topped with foil.

\(^a\) Pestles: 1.5ml Pestle, (*100) / VWR International/ Article no. 431-0094
\(^b\) Cordless pestle motor: Motor*1 (+100*pestles) / VWR International / Article no. 431-0100
\(^c\) Sarcosil: N-Lauroylsarcosine sodium salt (500g) / Sigma-Aldrich / Article no. L5125

Figure 10. Left- All equipment necessary for grinding. Right - Pestle motor used for grinding
**12.2 Protocol: CS Elisa**

Protocol derived from Wirtz *et al.* (1989) and Stone *et al.* (2013) [37, 38]

**Materials:**
- Blocking buffer: 5% Dried skimmed milk in PBS
- Sterilin round bottom plates > Replaced by NUNC Immuno plates (Thermo Scientific, cat. nr. 439454)

<table>
<thead>
<tr>
<th>Name</th>
<th>Stock conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 3SP2</td>
<td>2mg/mL</td>
</tr>
<tr>
<td>Conjugate 3SP2 HRP</td>
<td>1mg/mL</td>
</tr>
<tr>
<td>Substrate TMB (tebu-bio, cat.nr. TMBW-1000-01)</td>
<td></td>
</tr>
<tr>
<td>Positive control CSP Gennova</td>
<td>2µg/mL</td>
</tr>
</tbody>
</table>

**Protocol:**

1. Grind mosquito as described under DNA extraction.
2. Add 100µl capture Mab to all wells. Leave for 3h at room temperature (RT).
3. Wash 3*in PBS.
4. Add 150µl blocking buffer. Leave for 1h at RT.
5. Wash 3*in PBS.
6. Mix mosquito homogenate well, allow to settle for a few seconds, add 50µL of the liquid to sample wells. Ensure that multiple blank wells (no homogenate) and pooled negative control wells (pooled homogenate from uninfected mosquitoes) are used on each plate as well as an 8 step (3-fold dilution) standard curve of CSP Gennova.
7. Incubate overnight at 4°C.
8. Wash 4*in PBS.
9. Add 100µL conjugate Mab to all wells. Leave for 3h at RT.
10. Wash 4*in PBS.
11. Add 100µL TMB. Leave for 20 min. at RT.
12. Add 50µL 0.2M H₂SO₄.
13. Read plate at 450nm.
12.3 Protocol: Sub-species determination by PCR

**Sub-species determination of the *An. gambiae* complex by PCR**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.375 µl</td>
<td>sterile H$_2$O</td>
</tr>
<tr>
<td>5.0 µl</td>
<td>Taq 5X PCR Buffer with MgCl$_2$</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>dNTP (10 mM mix)</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>MgCl$_2$ (25 mM)</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>UN (F, 25 pmol/µl) [GTGTGCCCCCTTCTCGATGT]</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>AR (R, 25 pmol/µl) [AAGTGTCCTTCTCCATCCTA]</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>GA (R, 25 pmol/µl) [CTGGTTTGGTGCGAAGTTT]</td>
</tr>
<tr>
<td>0.125 µl</td>
<td>Taq DNA polymerase (5 U/µl)</td>
</tr>
<tr>
<td>24 µl</td>
<td><strong>Total (To each 24 µl reaction add 1 µl 10x diluted template DNA)</strong></td>
</tr>
</tbody>
</table>


**PCR cycle conditions**

95°C/5min x 1 cycle  
(95°C/30s, 50°C/30s, 72°C/30s) x 30 cycles  
72°C/5min x 1 cycle  
4°C hold

Run samples on a 2% agarose EtBr gel; load 7 µl sample

Primers create fragments of 390bp *An. gambiae s.s* and 315bp *An. arabiensis*
12.4 Protocol: Detection of the knock-down resistance mutation detection

Protocol modified after Martinez-Torres et al. (1998) [40], Ranson et al. (2000) [41] and the MR4 manual [39].

**Kdr-west**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
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<tbody>
<tr>
<td>10.875 µL</td>
<td>Sterile H₂O</td>
</tr>
<tr>
<td>5.0 µL</td>
<td>5X PCR Buffer</td>
</tr>
<tr>
<td>0.5 µL</td>
<td>dNTP (10mM)</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD1 (2.5 pmol/µl) [ATAGATTCCCCGACCATG]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD2 (2.5 pmol/µl) [AGACAAGGATGATGAACC]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD3 (2.5 pmol/µl) [AATTGCATTACCTACGACA]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD4 (2.5 pmol/µl) [CTGTAGTGATGAAATTTA]</td>
</tr>
<tr>
<td>2.5 µL</td>
<td>MgCl₂ (25 mM)</td>
</tr>
<tr>
<td>0.125 µL</td>
<td>Taq DNA polymerase (5 U/ µL)</td>
</tr>
<tr>
<td>23 µL</td>
<td>Total (To each 23 µl reaction add 2 µl 10x diluted template DNA)</td>
</tr>
</tbody>
</table>

**Kdr-east**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
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<tbody>
<tr>
<td>10.875 µL</td>
<td>Sterile H₂O</td>
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<td>5X PCR Buffer</td>
</tr>
<tr>
<td>0.5 µL</td>
<td>dNTP (10mM)</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD1 (2.5 pmol/µl) [ATAGATTCCCCGACCATG]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD2 (2.5 pmol/µl) [AGACAAGGATGATGAACC]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD3 (2.5 pmol/µl) [AATTGCATTACCTACGACA]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD4 (2.5 pmol/µl) [CTGTAGTGATGAAATTTA]</td>
</tr>
<tr>
<td>1.0 µL</td>
<td>AgD5 (2.5 pmol/µl) [TTTCATTACCTACGACTG]</td>
</tr>
<tr>
<td>2.5 µL</td>
<td>MgCl₂ (25 mM)</td>
</tr>
<tr>
<td>0.125 µL</td>
<td>Taq DNA polymerase (5 U/ µL)</td>
</tr>
<tr>
<td>23 µL</td>
<td>Total (To each 23 µl reaction add 2 µl 10x diluted template DNA)</td>
</tr>
</tbody>
</table>

**PCR cycle conditions**

94°C/5min x 1 cycle  
(94°C/30s, 50.8°C/45s, 72°C/1min) x 40 cycles  
72°C/10min x 1 cycle  
4°C hold

Run samples on a 2% agarose EtBr gel; load 7 µl sample.  
Primers create fragments of 293 internal control, 195 resistant, 137 susceptible.
# 12.5 Field forms

**MSF-OCA MALARIA VECTOR STUDY, DRC- NORTH KIVU**

**CDC LIGHT TRAP FORM**

## 1.0 BASIC DATA

1.1. Province…………………….. **NORTH KIVU**

1.2. Name of district………………..

1.3. Name of health zone…………..

1.4. Village name …………………

1.5. House/room identification…………………………………………………..

1.6. GPS point (LAT)-Y-COORD…….. **LAT**

1.7. GSP point (LONG)-X-CORD……. **LONG**

1.8. Elevation …………………………………………………..

1.9. Date of mosquito collection……. **DCATCH**

## 2.0 HOUSE/ROOM CHARACTERISTICS

2.1. Type of house……….. 1. Indigenous house/household 2. Displaced house/household **HOUTYPE**

2.2. Type of walls………… 1. Mud 2. Brick 3. Cement/plaster 4. Wood/Sticks **WALTYP**

2.3. Type of roof…………. 1. Corrugated Iron/zinc sheets 2. Mud or Concrete roof 3. Thatch or grass roof **ROOFTYP**

2.4. Is this house sprayed in the past (IRS)?

Yes → When was the last time

2.5. Does the room have a CEILING? …………………………………………..

2.6. Are there EAVES present at edges of roof of the room/ house? …………..

2.7. Does the house (room) have ELECTRICITY? ……………………………..

2.8. How many DOORS does the room have? …………………………….

2.9. How many WINDOWS does the room have? ……………………………

## 3.0 Number of people that slept in the room the previous night

3.1. Number of males 0-5 years that slept in room the previous night……..

3.2. Number of females 0-5 years that slept in room the previous night…..

3.3. Number of males 6-18 years that slept in room the previous night…..

3.4. Number of females 6-18 years that slept in room the previous night …

3.5. Number of males above 18 years that slept in room the previous night……

3.6. Number of females above 18 years that slept in room the previous night…….
4.0 MALARIA PREVENTION METHODS (ONE WEEK PRIOR TO TODAY):

4.1. Has any insecticide spray been used in room one week before today? …… 1. Yes 2. No INSPIRAY
4.2. Has any mosquito coil been used one week before today? ……….. 1. Yes 2. No COIL
4.3. Has smoky fire been used to prevent mosquitoes a week before today? … 1. Yes 2. No SMOKE
4.4. Has strongly scented leaves been used one week before today? …….. 1. Yes 2. No SCENTL
4.5. Has mosquito repellent cream been used one week before today? ……. 1. Yes 2. No RCREAM

5.0 BED NET
5.1. Is there a mosquito net present in room? [Verify please]……………… 1. Yes 2. No BEDNET
5.2. Is/Are the mosquito net(s) insecticide treated ones (LLINs)? ……… 1. Yes 2. No 9. NA NTREAT
5.4. Is/Are the mosquito net(s) pierced or torn? ……………………. 1. Yes 2. No 9. NA NETTORN
5.6. At what time do they enter their hut in the night? ………………… 1. Yes 2. No 9. NA TENTER
5.7. At what time do they go to sleep? ………………………………… 1. Yes 2. No 9. NA TSLEEP

6.0 LIGHT TRAP COLLECTION OF MOSQUITOES
6.1. What time was light trap set [GMT format]? …………………… 1. Yes 2. No 9. NA LSET
6.2. What time was light trap removed [GMT format]: ……………… 1. Yes 2. No 9. NA LMOVE
6.3. Was the light (of trap) on till the next morning? …………………… 1. Yes 2. No LIGHTON

7.0 ANOPHELES GAMBAE SPECIES
7.1. Number of UNFED (UF) females caught in trap…………………… 1. Yes 2. No UFGAMB
7.2. Number of BLOODFED (BF) females caught in trap……………….. 1. Yes 2. No BFGAMB
7.3. Number of HALF-GRAVID (HG) females caught in trap……………. 1. Yes 2. No HGGAMB
7.4. No of GRAVID (G) females caught in trap…………………………. 1. Yes 2. No GGAMB

8.0 ANOPHELES FUNESTUS SPECIES
8.1. Number of UNFED (UF) females caught in trap…………………… 1. Yes 2. No UFFUNES
8.2. Number of BLOODFED (BF) females caught in trap……………….. 1. Yes 2. No BFFUNES
8.3. Number of HALF-GRAVID (HG) females caught in trap……………. 1. Yes 2. No HGFUNES
8.4. Number of GRAVID (G) females caught in trap…………………… 1. Yes 2. No GFUNES

9.0 ANOPHELES PHOROENSIS SPECIES
9.1. Number of UNFED (UF) females caught in trap…………………… 1. Yes 2. No UFPHAR
9.2. Number of BLOODFED (BF) females caught in trap……………….. 1. Yes 2. No BFPHAR
9.3. Number of HALF-GRAVID (HG) females caught in trap……………. 1. Yes 2. No HGPHAR
9.4. Number of GRAVID (G) females caught in trap…………………… 1. Yes 2. No GPHAR

10.0 ANOPHELES RUFIPES SPECIES
10.1. Number of UNFED (UF) females caught …………………………… 1. Yes 2. No UFRUFIP
10.2. Number of BLOODFED (BF) females caught ……………………… 1. Yes 2. No BFRUFIP
10.3. Number of HALF-GRAVID (HG) females caught …………………… 1. Yes 2. No HGRUFIP
10.4. Number of GRAVID (G) females caught in trap

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<td></td>
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<td>GRUFIP</td>
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### 11.0 ANOPHELES COUSTANI

11.1. Number of UNFED (UF) females caught

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<td>UFCOUS</td>
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11.2. Number of BLOODFED (BF) females caught

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<td>BFCOUS</td>
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11.3. Number of HALF-GRAVID (HG) females caught

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<td>HGCOUS</td>
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11.4. Number of GRAVID (G) females caught

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<td>GCOUS</td>
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### 12.0 ANOPHELES MOUCHETI

12.1. Number of UNFED (UF) females caught

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<td></td>
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<td>UFMOUC</td>
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12.2. Number of BLOODFED (BF) females caught

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<td>BFMOUC</td>
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12.3. Number of HALF-GRAVID (HG) females caught

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<td>HGMOUC</td>
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12.4. Number of GRAVID (G) females caught

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<td>GMOUC</td>
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### 13.0 CULEX SPECIES

13.1. Number of UNFED (UF) females caught

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<td></td>
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<td>UFCULEX</td>
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13.2. Number of BLOODFED (BF) females caught

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<td>BFCULEX</td>
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13.3. Number of HALF-GRAVID (HG) females caught

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<td>HGCULEX</td>
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13.4. Number of GRAVID (G) females caught

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<td>GCULEX</td>
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### 14.0 ADEDES SPECIES

14.1. Number of UNFED (UF) females caught

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<td>UFAEDES</td>
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14.2. Number of BLOODFED (BF) females caught

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<td>BFAEDES</td>
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14.3. Number of HALF-GRAVID (HG) females caught

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<td>HGAEDES</td>
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14.4. Number of GRAVID (G) females caught

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<tr>
<td></td>
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<td>GAEDES</td>
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</table>

### 15.0 MANSONIA SPECIES

15.1. Number of UNFED (UF) females caught

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<tr>
<td></td>
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<td>UFMAN</td>
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15.2. Number of BLOODFED (BF) females caught

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<td>BFMAN</td>
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15.3. Number of HALF-GRAVID (HG) females caught

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<td>HGMAN</td>
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15.4. Number of GRAVID (G) females caught

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<td></td>
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<td>GMAN</td>
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### 16.0 PHLEBOTOMES (SANDFLIES) SPECIES

16.1. Number of UNFED (UF) females caught

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16.2. Number of BLOODFED (BF) females caught

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16.3. Number of HALF-GRAVID (HG) females caught

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<td>HGSAND</td>
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16.4. Number of GRAVID (G) females caught

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<tr>
<td></td>
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<td>GSAND</td>
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</tbody>
</table>
1.1. Province…………………………. NORTH KIVU
1.2. Village name ...........................

1.3. Date of bed net collection.......  
1.4. Type of bed net …  
1.5. Age of the bed net...........

1.6. How many times do they wash the bed net? ...............

1.7. Do they dry the bed net directly in the sun light?

1.8. Do you know why you need a bed net?

---

1.1. Province………………….…..  NORD KIVU
1.2. Nom du village ............................

1.3. Date de collection de la moustiquaire  
1.4. Type de moustiquaire  
1.5. Age de la moustiquaire  .........

1.6. Combien de fois avez-vous déjà lessivé la moustiquaire? ...............

1.7. Séchez-vous directement la moustiquaire au soleil après lessive?

1.8. Savez-vous l'importance de la moustiquaire?