

The First GLP Compliant Study in Africa for the Evaluation of LLIN's; Efficacy of SafeNet® and SafeNet NF®

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Research Article

Keywords: Good Laboratory Practice, universal coverage

Posted Date: November 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1006810/v1>

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Abstract

Background: To attain and sustain the universal Long-Lasting Insecticidal Nets (LLINs) coverage, cheap nets that provides equivalent or better protection than the standard LLINs, are required. While it is essential to follow the World Health Organization (WHO) guidelines for the evaluation of LLINs, adherence to the Good Laboratory Practice (GLP) is necessary to generate reliable and reproducible data that will facilitate efficient LLINs to be speedy registered. Adherence to GLP obviate the need to duplicate the assessment and ensures substandard LLINs are not reaching the market. This study aimed to evaluate efficacy of SafeNet NF[®] and SafeNet[®] LLIN in accordance to the WHO Pest Evaluation Scheme (WHOPES) and the GLP guidelines. Both candidate LLINs were manufactured with less fabrics to cut down manufacturing costs, motivated by the need for cheaper LLINs to achieve universal coverage.

Materials & Methods: SafeNet NF[®] and SafeNet[®] LLIN, were assessed in experimental huts against wild, pyrethroid-resistant *Anopheles arabiensis* mosquitoes. Efficacy in terms of mosquito blood-feeding inhibition and mortality, was compared with Interceptor[®] LLIN and an untreated net. All nets were washed and artificially holed to simulate a used torn net. The GLP guidelines were followed throughout this study.

Results: The mortality of mosquitoes exposed to SafeNet NF[®] and SafeNet[®] LLIN were equivalent to that of the reference net. Blood-feeding inhibition was only evident in Interceptor[®] LLIN. Adherence to GLP was observed throughout the study.

Conclusions: Step-wise procedures to conduct LLIN evaluation in compliance to both WHOPES and GLP guidelines are elaborated in this study. SafeNet NF[®] and SafeNet[®] LLIN offers equivalent protection as Interceptor[®] LLIN and can facilitate universal LLIN coverage due to its low manufacturing cost. However, further research is needed to understand durability, acceptability and residual efficacy of these nets in field environments.

Background

Long-lasting insecticidal nets (LLINs), are the main tool for malaria vector control in sub-Saharan Africa, and have contributed to the 75% reduction in the global burden of malaria in the recent years [1]. To maximize the impulse of disease reduction, and in response to the United Nations (UN) Secretary General call for a scale-up of Insecticide-treated bed nets (ITN) coverage, global LLIN deliveries reached approximately 250 million LLINs in 2019 [2]. Despite this effort, global LLIN coverage remains inadequate, with 44% of the population in endemic areas estimated to be lacking access to LLIN [3]. The World Health Organization (WHO), in the 2017 update of its recommendations on achieving universal coverage with LLINs, defines “universal coverage” as “universal access to, and use of, LLINs” for the entire population at risk of malaria targeted in the control or elimination strategy [3]. Long-lasting insecticidal nets are one of two main vector control interventions suggested for universal coverage by the WHO [4], leading to the massive LLINs production in various parts of the world. Lack of strict monitoring during production and evaluation has resulted into substandard LLIN production consequently resulted into purchase of 52 million faulty Long-Lasting Insecticide-treated Nets (LLIN) worth \$106 million from 2017 to 2019 (6). To avoid this, the vector control group of the WHO Prequalification Team of Vector Control Products (WHO PQT-VC) was mandated to cooperate with national regulatory agencies and partner organizations, to ensure effective and high-quality vector control products are accessible to those who urgently need them [5]. As a consequence, from 2017 to 2020, the WHO PQT-VC has assessed and qualified 21 LLIN brands, which are acceptable for procurement by UN and other international agencies or countries [5]. Furthermore, the WHO PQT-VC emphasizes the need for test facilities involved in the evaluation of mosquito control products to comply with GLP [6]. Enhancing this capacity for vector control product evaluation ensures generation of quality data which in turn facilitate speedy registration of products to the market.

While across the African continent, there is fluctuating pattern of coverage, caused by nets wearing out [7], gains in malaria control have stalled, and less than 50% of endemic countries remain on track to reach critical malaria reduction targets [2]. Furthermore, the global investment for malaria has shrunk, with a witnessed \$ 2.3 billion less, equivalent to 50% financial shortage to meet the WHO target to reduce malaria incidence and mortality by 40% by the year 2020 [8]. This has raised concerns about the future of LLIN coverage, and a renewed interest in looking for additional private sector investment to finance, produce and deliver cheaper and affordable LLINs.

The objective of this study was to compare the efficacy of SafeNet NF[®] and SafeNet[®] LLIN in terms of mortality and blood-feeding inhibition to the reference Interceptor[®] LLIN, in accordance to WHOPES guidelines and the GLP standards.

Materials & Methods

Study Site

The field evaluation of the LLINs was conducted in the East African style experimental huts, located at Pasua (S03°22.764' E03°720.793'), in Lower Moshi adjacent to Lower Moshi rice irrigation scheme (**Figure 1**). The irrigation scheme is getting water from the Rau river catchment, providing reliable breeding site for local malaria vectors, *Anopheles arabiensis*. The *An. arabiensis* in this area has moderate pyrethroid resistance due to elevated levels of both mixed function oxidases and β -esterases [12,13].

The GLP procedures

This study adhered to the following procedures to ensure GLP compliance, summarized in the study flow **Figure 2**:

Pre-study meetings

Before study initiation, a series of communication meetings with the sponsor were conducted to ensure; contract agreements (evaluation terms) are approved and signed, agreement on shipping of test items, type of reference nets, documentations (Safety Data Sheet, Certificate of Analysis), agreement to submit study plan to WHO PQT-VC for review, evaluation in accordance to shared Standard Operating Procedures (SOPs), WHOPES guidelines and GLP standards, confirmation of regeneration time for the test items, identification of GLP certified test sites for chemical analysis, key information for the study plan, timely submission of the study plan draft to sponsor and review of the study at testing site.

Parallel to this, a meeting was held at the Test Facility by the Test Facility Manager to appoint a responsible Study Director (SD) for this study. The Test Facility Management ensured that the facility had sufficient space, infrastructure, man power and materials to accommodate the study.

Identifying subcontractor for HPLC analysis

The Test Facility (TF) identified and shared with the sponsor (manufacturer); names, quotes and address of the two identified GLP compliant test sites for conducting the High-performance liquid chromatography (HPLC) for chemical analysis. One test site, BioGenius GmbH (Germany) was chosen for doing chemical analysis, based on the availability of Test Site (TS) to do the work that fits our agreed timeline, GLP certification, and cost of the quote.

Contracts

The contracts were established and signed covering: definitions, terms, performance of evaluation and reporting, confidentiality of test items, intellectual property rights, publication, payments, liability, force majeure, miscellaneous and sections for signing both parties. A similar but separate contract was also signed between Test Facility and the Test site. Along with the contract, the budget and quotes were submitted to the sponsor.

Study plan and Implementation plan

Standard format for study plan [14,15] was used with considerations to the OECD GLP guidelines [16] to establish a study plan that describe the aim, test items, test systems, experimental design and statistical analyses for the study. The established study plan was used to inform all participants on the study requirements and the timing of events to ensure that these are fulfilled to the mutual satisfaction of all parties. The study plan was also shared with the WHO PQT-VC for review and recommendations. Implementation plan was established to indicate a cascade of events in a chronological order to meet study objectives. Both implementatin and study plans were prepared by the TF and shared with sponsor and TS forreviewand agreement before actual implementation.

Study opening and the master schedule

Once all contracts, study plans and quotes were reviewed (with inputs from WHO PQT-VC, sponsor and test site) and signed, a study specific folder was opened to document all descriptive and prescriptive information related to the study, and a study was given a unique code. All the data collection forms and other forms related to this study were using this unique code.

During monthly master schedule meetings, the study was introduced to the senior management for record in the “master schedule”, which is a meeting to record the phases and dates on which various studies pass through a series of phases from planning until archiving [17] (**Figure 3**). In this meeting, study director, Test Facility Management, project manager and data manager. At the first master schedule meeting, number and type of forms were communicated to data manager to establish a necessary database for data entry.

Study initiation and experimental phases

Once the WHO PQT-VC recommendations were incorporated and considered in study plan, the study plan was signed by study director, Test Facility Manager, Test site Manager, TF Quality Assurance (QA) Manager and the TS QA Manager. A kick-off meeting was conducted at the TF, involving all the study participants. In that meeting, Study Director (SD) introduced the study, test items, test systems and the overview of the relationship between the TF and TS and the study plan was explained in detail. Experimental date was announced, all data collection forms that were to be used, relevant SOPs for this study, Safety Data Sheets (SDS) were discussed.

Study risk assessment and mitigation

Study plan was submitted to the Health and Safety officer to identify any potential risk associated with conducting the study and to recommend measures to be taken in order to minimise health risks and to guide study personnel in the study procedures. In Tanzania, the risk assessment is a requirement by law [18].

Quality assurance auditing for study plan and identification of critical phases

To ensure that, the procedures being conducted were of quality, and the results are trusted, the lead Quality Assurance (QA) Manager from the TF and the QA Manager from the TS were involved for the study plan auditing. The identified non-conformances were corrected by the Study Director and the Project Manager. Furthermore, the Quality Assurance Manager, Project Manager and Study Director met and discussed the critical phases that would be most appropriate for QA audits. It was agreed that the net washing, should be first to be audited since it impacts directly on the test items. The 10th wash was chosen for auditing because there would be sufficient records as well as observing the practical aspect of washing according to SOPs. Another element of the experimental phase that was audited was cone bioassays because they directly assess test items. The aspects of hut trials were agreed for auditing include, rotation of treatments, collection of mosquitoes and scoring of primary outcome measures. The critical phases were identified by Test Site QA and Principal Investigator and was included in the audit report. At the TS, it was agreed that the experimental determination of alpha-cypermethrin content in long-lasting insecticidal must be audited.

Data management, Software validation, and accuracy checks

The study director discussed with the data management team the forms to be used in this study for net cone bioassays, mosquito collection, test system biometrics, bottle bioassays and mosquito packing. The data entry procedure, databases preparation, computers and software validation SOPs, comparison and accuracy check plans for the study were reviewed and discussed.

Record of procedures

In this GLP study, the record of procedures, a form that keeps track of specific actions that involve the test items in the experimental procedures was used throughout the study duration (May 2019 to December 2019). Recorded experimental procedures include: cutting net pieces, washing nets, cutting holes on nets, collection of test systems during hut trial, scoring of immediate and delayed mortality, packing of test systems, cutting of net pieces for bioassays, conduct of bioassays, scoring of bioassay outcome measures, shipment of test items and archiving of test items. In the record of procedures information on

equipment used, test items, test systems, technician initials and date were recorded. Sequence of events, equipment used and staff qualification involved in the experimental phase were among essential records that were used by our external auditor, South African National Accreditation System (SANAS).

Final report

The final report was written and signed by SD, lead QA, TFM and sponsor.

Archiving

At the end of the study, the original study folder, containing; the study plan, schedule of activities, correspondence with sponsors, amendments & deviations, records of procedures, the raw data, ancillary study data, analysis print-outs and final reports, was archived at the test facility for a period of five years in accordance with internal SOPs after agreement with the sponsor. Also, one unwashed unused net of each treatment was retained in the Test Facility and archived for one year.

All used or expired test items were disposed with the sponsor's consent at the conclusion of the study. The dried carcasses of the test systems analysed in the molecular laboratory are being archived for a period of five years in accordance with internal SOPs.

Waste disposal

All waste generated in the course of the study were disposed in accordance to the OECD GLP document number 19 [19], the Test Facility waste disposal SOPs and recommendations from the National Environmental Management Council of Tanzania (NEMC).

Experimental evaluation of SafeNet[®] and SafeNet NF[®] LLIN

Test items

The test items for the current study were SafeNet[®] and the 'SafeNet NF[®]' LLINs; Piperonyl butoxide (PBO) and alphacypermethrin (ACM). The LLINs are polyester nets coated with alphacypermethrin. The difference between the two candidate nets is the physical characteristics, with SafeNet NF[®] having wider mesh, lower grams per square meter (GSM), higher denier, lower bursting strength, **Table 1**.

Technical grade insecticides for supplementary bioassays

The working solutions of Piperonyl butoxide (PBO, batch number 8615500) and alphacypermethrin (ACM, batch numbers 5823400 and 8592500) were prepared from technical grade received from a commercial supplier (Chem Service Inc. West Chester PA, USA). The purity of the first sample was reported as 98.4%±0.5%, and the second sample as 99.5%±0.5%, by an ISO-certified testing facility.

The purity of this test item was reported as 98.3% ±0.5% as indicated on the certificate of analysis by an ISO-certified testing facility. The certificates of analysis were considered sufficient verification of integrity and quality of the technical grade insecticides.

Characterization of test systems

Bottle bioassays, biometric tests, and molecular assays were conducted to characterize mosquitoes that were used for the experimental hut trial and laboratory bioassays.

Test systems for experimental hut trial: The wild population of *An. arabiensis* in Lower Moshi were caught at larvae stage and reared to adults for use in bottle bioassays. In total, 300 female unfed 2-5day old adult mosquitoes were tested against alphacypermethrin with the 1x and 5x diagnostic dose bottle bioassays as per CDC guidelines [20], and 150 in the pre-exposure bottle bioassay (including 50 for the controls).

Test systems for cone bioassays: *An. gambiae* s.s. Kisumu, a fully-susceptible strain was used. Unfed 2-5d old adult females from the insectary were characterised in terms of body weight, wing length, resistance status (phenotypic and genotypic) and species identification, during the experimental phase of the study.

A total of 88 *An.gambiae* s.s. (Kisumu strain) mosquitoes were tested for species identification and *kdr E* genotype using quantitative real time qPCR technique. DNA was extracted from the *Anopheles spp* using the modified chelex extraction method by Walsh [27]. Identification of the members of the *An.gambiae* s.l. species complex was performed using the Taqman 3-plex assay of Bass *et al* [22]. Detection of *kdr* mutations was performed using the Taqman assay method [21]. A separate sample of 100 *An. gambiae* s.s. Kisumu were used for the biometric characterisation of the colony following the modified methods by Yeap [23] and Nasci [24].

Washing and preparation of LLINs for field trial

Whole nets were washed in accordance with WHOPES guidelines [25]. In brief, each net was washed in Savon de Marseilles soap solution for 10 minutes: 3 minutes stirring, 4 minutes soaking, then another 3 minutes stirring. This was followed by 2 rinse cycles of the same duration with tap water only. The mean water pH was 6 for all washes. The mean water hardness was 50.4 parts per million (ppm) and always within the WHOPES limit of ≤ 89 ppm. Seven nets of each treatment (**Table 1**) were washed for use in the hut study, and one additional net was washed and retained/'held back' (HB), which is not used in the hut study (**Table 2**). All nets used in the experimental hut study had 30 holes (4x4 cm) cut in them to simulate the conditions of a torn used net.

Five pieces 30x30 cm were cut from nets held back (one net for each treatment arm) before and after they were washed, 10 pieces in total per net (HB0-HB9). At the conclusion of the hut trial, hut used (HU) nets were returned to the Test Facility and five pieces (HU1-HU5) were cut from one net from each treatment arm, chosen randomly. In all instances, pieces were cut from pre-defined positions on the nets. Pieces were wrapped in aluminium foil, and kept in a fridge in test room 2 at $5\pm 3^{\circ}\text{C}$ until needed for assays.

Preparation of bottle bioassay working solutions

Four bottles of ACM at 12.5 $\mu\text{g}/\text{mL}$ were prepared for testing in a single test, and four bottles of ACM at 60 $\mu\text{g}/\text{mL}$ for a separate assay test. The bottles were coated evenly following CDC Bottle Bioassay guideline [16]. Four additional Wheaton bottles were coated with 1mL acetone only; these were used as the negative controls. The PBO bottles were prepared in the same way, from which a dilution in acetone to 25 $\mu\text{g}/\text{mL}$ was prepared. One mL of this dilution was used to coat each of 3 Wheaton bottles.

All stock and working solutions were used within 24 h of preparation. Stock solutions were diluted immediately to create the working solutions, which were immediately used to coat the bottles. Likewise, once treated the bottles were used within 5 days.

Running the experimental hut trial

The experimental hut study was conducted from June 2019 to August 2019 at Pasua Field Station in seven huts, with treatment arms as indicated in **Table 2**.

In brief, treatments and baits (cows used to attract mosquitoes into experimental huts) were randomized using a 7X7 Latin square design (<https://www.dcode.fr/latin-square>; accessed 20 May 2018). The treatments were rotated weekly, with daily rotation of candidate nets of the same treatments, while the cows were rotated daily. Cows were kept inside the experimental huts for 12 hrs (from 6:30pm to 6:30am), and collection of mosquitoes was conducted in the morning. Mosquitoes were collected daily from the window exit traps, verandahs, room and inside the net from each of the 7 huts. Both live and dead mosquitoes were collected in separate cups for live, blood fed, unfed, dead and their point of location recorded. All mosquitoes caught alive were kept in paper cups provided with glucose solution 10%. Mortality was scored 24 h later.

Supplementary cone bioassays

Cone bioassays for pieces cut from hut trial's used and unused net were conducted in accordance to the standard WHOPES guidelines [26]. In brief, 2 replicates for each of the 5 pieces per net were run with 5 mosquitoes per cone, making a total of 50 mosquitoes for each net representing each treatment arm. Negative control pieces were tested alongside the test items and reference item. Any replicates for which control mortality exceeded 10% at 24 h were not analysed, and further replicates were carried out to replace those that were excluded. For all bioassays, there was a minimum of one hour holding period pre-exposure and a 3-minute exposure time. After exposure, mosquitoes were released into holding cups and provided with 10% glucose solution, 60 minutes knockdown and 24h mortality were recorded.

Supplementary bottle bioassays

The CDC Bottle Bioassay guideline [20] was followed for both a) the ACM-only bioassays and b) the PBO pre-exposure bottle bioassays. In brief, in the ACM-only assays mosquitoes were exposed directly to ACM 1x or 5x (4 bottle of each concentration, 25±3 mosquitoes/bottle). In the PBO pre-exposure assays mosquitoes were exposed to acetone and PBO (25 µg/mL) for 1 hour, then held in cages for 1 hour before exposure to ACM 1x (4 bottles, 25±3 mosquitoes/bottle) and acetone (2 bottles, 25±3 mosquitoes/bottle).

During the 30 min exposure to ACM or acetone, knockdown was recorded at 0 minutes and then every 5 minutes until 30 minutes. After 30 minutes, mosquitoes were removed from the bottles, transferred back into the holding cups, and provided with glucose solution 10%. At 24h post-exposure mosquitoes were scored as dead or alive.

Data analysis

The number of mosquitoes per bottle and cones were within the acceptable range of 25±3 and 5±1 mosquito respectively. The control mortality for cones and bottle bioassays were all less than 10%, therefore mortality in the insecticide treatment groups were not adjusted.

The 95% confidence intervals for proportionate data were calculated from the proportion of observations of interest (number knockdown/number dead), the total observed, assuming $\alpha = 0.05$, using the following formula:

$$\text{confidence interval} = \sqrt{\left(\frac{\text{proportion} \times (1 - \text{proportion})}{\text{total observed}}\right)} \times 1.96$$

Double entry, comparison checks and accuracy checks on all the datasets were carried out in MS Access 2016. All datasets were transferred into Stata I/C v11.0 (StataCorp LLC, Texas USA) statistical software using Stat Transfer v8.0. Graphs were created in MS Excel 2016.

The data for the free-flying mosquito collections in the hut trial were analysed using logistic regression for grouped data with odds ratio output, adjusting for effects of hut and sleeper. Statistics were run using Stata/IC version 11.1 (StataCorp LP College Station TX77845, USA). The main outcomes from the experimental huts were: deterrence, blood-feeding inhibition, and mortality. The 2 sample t-test was used to compare the mortality data between candidate net and reference nets at both 0 and 20 times washes for the pieces derived from hut used nets.

Results

GLP Procedures:

Study risk assessment and mitigation

Activities with potential hazards identified were; net (pieces and whole) washing, bioassay and field trial. Likelihood and severity were scored as 2 and 1 respectively, see scale (28) used **Table 3**.

Persons potentially at risk were identified. Gloves, gumboot, overalls were recommended as appropriate personal protective equipment for whole net washing while lab shoes, lab coat and gloves were recommended for use during laboratory bioassays. The overall risk score for the three study activities assessed was 1, which implies low risk based on 0-5 scale.

Critical phase auditing

The 10th wash was audited, the QA manager found that the 10th and all previous washing from gathered records complied with SOPs and study plan. Furthermore, the conduct of cone assays and the rotation of treatments and the collection of mosquitoes and scoring of primary outcome measures during the hut trial were found to conform with SOPs and study plan. Equivalently, the audit of the experimental determination of alpha-cypermethrin in long-lasting insecticidal was found complying with SOPs and study plan at the test site.

Data management, software validation, and accuracy checks

The data manager reviewed the maintenance records and validation of excel and Stata software on all computers that were intended to be used for the study. The SD and data management team were satisfied that everything is in place and fit for purpose before study experimental phase. Microsoft Access database for data entry was created and validated.

Record of procedures

From May 2019 to December 2019 all practical activities involving the test item such as: cutting net pieces, washing nets, cutting holes on nets, collection of test systems during hut trial, scoring of immediate and delayed mortality, cutting of net pieces for bioassays, conduct of bioassays, scoring of bioassay outcome measures, shipping of test items to Test Site and archiving of test items were documented in the record of procedures form.

Study compliance to GLP

The study was audited internally and externally by SANAS as part of the annual audit inspection of the Test Facility and no serious study-related non-conformances were found. This study complied with the conditions and requirements of the South African National Accreditation System (SANAS), which are in accordance with the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (revised, 1997) in the conduct of non-clinical health and environmental safety studies of test items contained in pharmaceutical products, veterinary drugs and pesticides. The test facility is accredited to conduct studies to such standards since 2017 [29].

Experimental hut study:

One thousand and forty-seven (1047) free-flying *An. arabiensis* were collected from the experimental huts on 49 nights from June to August 2019.

Mosquito entry rates in the huts with Interceptor® LLIN were reduced compared to negative control hut (Safi net). In the Interceptor® LLIN 0W arm there was 46.4% deterrence and 34.1% in the 20-times washed (20W) arm. Deterrence was not observed for any other arms of the trial (Table 2). Similar exiting rates were found for all the treatment arms and no significant induced exiting was detectable because the exiting rate in the control hut was very high, - 89.4%. There was evidence of blood feeding inhibition in the Interceptor® LLIN treatment groups (0W and 20W) but not in other arms. In the control hut, the proportion of blood fed female mosquitoes was 30.6% compared to the total number captured. For the Interceptor® LLIN 0W and 20W, blood feeding inhibition was 42.5% and 35.8% respectively, though after adjustment for the effects of hut and cow the latter was found to be not significantly different.

All insecticide treatments induced significantly higher 24 h mortality compared to the negative control (Table 2). In a comparison of the 6 insecticidal treatments with each other, the performance of SafeNet NF® was not statistically different from the reference arm (0W and 20W Interceptor® LLIN). SafeNet® LLIN 0W had an equivalent performance to Interceptor® LLIN 0W and 20W; however, SafeNet® LLIN 20W performed marginally lower (24 h: OR 0.52 (0.28-0.98), Z=-2.01, p<0.044). In **Table 4**.

Bloodfed % treatments sharing superscript letters are not significantly different from each other.

Cone assays for net pieces

The mean knockdown and/or 24 h mortality induced by net pieces, cut from all insecticide-treated nets 'held back' and 'unused' from the hut study passed the standard WHOPES cut-off criteria (either 24 h mortality $\geq 80\%$ or knockdown $\geq 95\%$ or both), at either unwashed and/or washed 20 times, where Interceptor® unwashed (mortality 100%), Interceptor® 20Xwashed (Kd 98%), SafeNet NF® unwashed (mortality 100%), SafeNet NF® 20Xwashed (Kd 100%), SafeNet® LLIN unwashed (Kd 100%, mortality 98%), SafeNet® LLIN 20Xwashed (Kd 96%) (**Fig 4., Table 2**). SafeNet® LLIN 20W and SafeNet NF® 20W induced higher mortality than the reference net at 20W, **Table 5**.

The efficacy of the 20W pieces for all treatments appeared lower than their unwashed counterparts. Nevertheless, these pieces still induced very high knockdown rates (all above 95%). In total, 798 females unfed 2-5-day old *An. gambiae s.s.* Kisumu mosquitoes were exposed in the cone bioassays: 398 for pieces from used net ('HU' pieces) and 400 for unused net ('HB' pieces).

mean knockdown and 24 h mortality induced by net pieces cut from randomly-selected insecticide-treated nets used in the hut trial also passed the standard WHOPES cut-off criteria. The 0W and 20W hut-used SafeNet® LLIN and SafeNet NF® passed both the knockdown and mortality cut-offs. The Interceptor® LLIN 0W passed on knockdown but failed 24h mortality (**Fig 5., Table 6**).

Bottle bioassays

All *An. arabiensis* exposed to 12.5 µg/mL ACM were knocked down within 30 minutes, but then showed recovery post-exposure: average mortality at 24 h was $35.0 \pm 9.3\%$ ($\pm 95\% \text{C.I.}$). This suggests the pyrethroid-resistance status of the wild caught *An. arabiensis* as per WHO criteria [2]. A similar result was seen with exposure to 5x diagnostic dose (60 µg/mL ACM). All mosquitoes (102) were knocked down within 25 minutes (Fig. 6a) but only 82.4% ($\pm 7.4\%$) were dead at 24 h (Fig. 6b.). This reflects metabolic resistance which was previously known about this test system [12] and was reconfirmed in subsequent PBO bottle bioassays and molecular lab assays during this study.

For the *An. gambiae* Kisumu strain; 24 h post-exposure mortality after a 30-minute exposure to ACM at 12.5 µg/mL was 100% in all three assays. This confirms the pyrethroid-susceptibility status of the test system. Susceptibility is demonstrated by a mortality of $\geq 98\%$ [26]. 24h post-exposure mortality after a one-hour exposure to Mero®-acetone vehicle only was always $<20\%$ - the maximum value was 17.4%.

Synergist bottle bioassays

The results for the 24 h mortality for the *An. arabiensis* against 12.5 µg/mL ACM without synergist pre-exposure and with synergist pre-exposure to PBO were 35% (with control adjusted mortality) and 100% respectively (**Fig 7**). This indicates the presence of metabolic resistance mechanism(s), involving overexpression of coded P450 genes.

Molecular characterisation of the test system

Results indicates that 100% of mosquitoes used for the cone bioassays were *An. gambiae s.s.*, and were all homozygous susceptible, *SSe*, meaning no mosquito was homozygous or heterozygous for the *kdr*L1014S gene. This further indicates that there was no evidence of strain contamination and that the strain was confirmed suitable for cone bioassays. Eighty-eight of the wild caught mosquitoes were assayed in the same way: 100% were shown to be *An. arabiensis* and no individuals were homozygous or heterozygous for the *kdr*L1014S gene, which means all samples were *SSe* status.

In all species identification assays all *An.gambiae s.s.* control and *An. arabiensis* control gave the expected amplification profile and the negative control gave no amplification. In all *kdr* assays all controls amplified correctly, that is *RRe* control, *RSe* control and *SSe* gave the expected amplification profile and the negative control gave no amplification, Table 7.

Biometric characterisation of the test system

The mean \pm 95% confidence interval for wing length for 50 *An. gambiae* s.s. Kisumu was 3.15 mm (3.11-3.19 mm). The mean \pm 95% confidence interval for body weight was 1.21 mg (1.13-1.28 mg). These means for the biometric measurements are within the 25-75% interquartile range of those data generated for from routine biometric screening of the strain (see Fig 8.), therefore the *An. gambiae* s.s. Kisumu used in this study were suitable for bioassays.

Discussion

The conduct of the multi-site GLP compliant study on the evaluation of LLINs in an African context is elaborated, with special considerations in the practical aspects. In this study it was found that delays caused by the multi-site nature of the study features a critical challenge. This is mainly attributable to the lack of appropriate equipment or collaborative research centres with qualifications to do chemical analysis in country, highlighting the need for capacity strengthening for the African research centres [30, 31].

The efficacy in experimental huts of the new ACM net, SafeNet NF®, expressed as 24h mortality of mosquitoes, was similar to that of the reference Interceptor® LLIN and consistent with the results anticipated for a pyrethroid-only net used in a setting where the predominant vector species, *An. arabiensis*, expressed metabolic resistance to pyrethroids but no knockdown resistance with a high exophilic behaviour. For SafeNet® LLIN, the performance was as good as the reference net unwashed but marginally inferior at 20x washes in the hut trial. As none of the insecticide treatment arms induced 30% mortality against the pyrethroid-resistant wild type mosquitoes, it is debatable whether any of the marginal statistically-significant differences detected between the treatment arms are biologically or epidemiologically relevant.

In the cone bioassays, pieces cut from unused nets and hut-used candidate nets passed either of the performance thresholds laid down by WHO [25], which are 24 h mortality $\geq 80\%$ and knockdown $\geq 95\%$, against the pyrethroid-only *An. gambiae* s.s. Kisumu strain. This was the case for both unwashed and 20x washed pieces. Recent witnessed shrinks in the investment for control of malaria is further impaired by the COVID-19 pandemic which has disproportionately affected the donor countries [9] causing a negative effect on malaria and other disease control programmes [10, 11]. Therefore, taking a stance to generate new LLINs at reduced manufacturer production costs could help on reaching and sustaining the LLIN universal coverage.

Conclusions

This study demonstrated that GLP-compliant evaluation of vector control products can be successfully carried out by African research institutions. However, more investments are needed to accommodate GLP-compliant GC-HPLC capacity within African research institutions participating in vector control product evaluation. Such investment will evade high cost to abroad contracted facilities, delays, and communication-associated challenges.

It was found that the bio-efficacy and wash resistance of both SafeNet® LLIN and SafeNet NF® are generally comparable to the reference product (Interceptor® LLIN). Equivalent performance with reference LLINs provides promise that these candidate nets can be considered to facilitate universal LLIN coverage. However, community or equivalent trial should be done to understand durability, acceptability and residual efficacy of these candidate nets in field context.

Declarations

Author Contributions: SA & MK; study conception and design, analysis and interpretation of results, drafted the first manuscript: JS; contributed to draft manuscript preparation, analysis interpretation and contributed to the final draft manuscript: RK; contributed to the final draft of the manuscript: JM; contributed to the final draft of the manuscript: OH; contributed to the final draft of the manuscript: MS; contributed to the final draft of the manuscript: KE; performed the experiments and contributed to the final draft of the manuscript: ET; performed the experiments and contributed to the final draft of the manuscript BGM; participated in the field experiments and contributed to the final draft of the manuscript BM; performed the laboratory bioassays and contributed to the final draft of the manuscript: FM; participated in the TF preparation for the study, legal contracts and contributed to the final draft of the manuscript. All authors have proof read the manuscript.

Funding: This research received payments from Mainpol GmbH for the LLIN evaluation.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Datasets generated and analysed are presented in a summarized way in this article, full datasets will be made available from the corresponding author upon rational request.

Acknowledgments: We thank the whole team at the KCMUCo-PAMVERC TF for their invaluable contribution to this study. We also thank the KCMUCo and KCMUCo-PAMVERC in particular for allowing me to do research using their facility and resources.

Conflicts of Interest: The authors declare no conflict of interest.

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Tables

Table 1. Characteristics for study LLINs

| Test Item | Active ingredient | Denier | GSM | Batch no. | Test Facility codes |
|--------------|--------------------------|---------------|---------------|------------------------|---------------------------------------|
| SafeNet® | 200mg/m ² ACM | 100 denier | 40±10% | NTG180702.1 | 749B-762B |
| | | | | WT.07.18.02 (7 nets) | 771B-772B |
| | | | | xxx20181020-1 (7 nets) | 785B |
| | | | | yyy20181020-2 (7 nets) | 788B-791B |
| SafeNet NF® | 200mg/m ² ACM | 100 denier | 36±10% | 456-20181020 (8 nets) | 735B-748B |
| | | | | 123-20181020 (7 nets) | 792B-798B |
| | | | | 789-20181020 (6 nets) | |
| Interceptor® | 200mg/m ² ACM | 100 denier | 40±10% | 4934415632 (21 nets) | 721B-734B 779B-784B 773B |
| Safi Net | N/A | Not indicated | Not indicated | No batch numbers | 763B-770B 774B-778B 786B & 787B |

Table 2. The experimental hut trial treatment arms

| Treatment arm | Washes | Used in Hut Trial | Nets held back |
|-------------------------|--------|-------------------|----------------|
| <i>Reference item</i> | | | |
| Interceptor® LLIN | x0 | 721B-727B | 727B |
| Interceptor® LLIN | x20 | 728B-734B | 731B |
| <i>Test items</i> | | | |
| SafeNet NF® | x0 | 735B-741B | 736B |
| SafeNet NF® | x20 | 742B-748B | 747B |
| SafeNet® LLIN | x0 | 749B-755B | 753B |
| SafeNet® LLIN | x20 | 756B-762B | 757B |
| <i>Negative control</i> | | | |
| Safi net | x20 | 763B-769B | 768B |

Table 3. Risks scores or likelihood and severity

| Likelihood | | Severity | |
|------------|------------------|----------|-----------------------------|
| 0 | Zero to very low | 0 | No injury or illness |
| 1 | Very unlikely | 1 | First aid injury or illness |
| 2 | Unlikely | 2 | Minor injury or illness |
| 3 | Likely | 3 | “3-day” injury of illness |
| 4 | Very likely | 4 | Major injury of illness |
| 5 | Almost certain | 5 | Fatality, disabling injury |

Table 4. Deterrence, exophily, blood feeding and mortality in the 7 treatment groups

ATTENT ARMS

| | Control | Interceptor® 0W | Interceptor® 20W | SafeNet NF® 0W | SafeNet NF® 20W | SafeNet® LLIN 0W | SafeNet® LLIN 20W |
|--------------------------------|-------------------|--------------------|--------------------|-------------------|--------------------|--------------------|-------------------|
| PERRENCY & EXOPHILY | | | | | | | |
| Females | | | | | | | |
| ght | 170 | 91 | 112 | 177 | 148 | 185 | 164 |
| errecy (%) | - | 46.5 | 34.1 | -4.1 | 12.9 | -8.8 | 3.5 |
| n | 11 | 5 | 9 | 22 | 19 | 22 | 17 |
| ndah dow + | | | | | | | |
| | 152 | 86 | 101 | 149 | 125 | 163 | 143 |
| | 7 | 0 | 2 | 6 | 4 | 0 | 4 |
| de net (%) | 4.1 | 0.0 | 1.8 | 3.4 | 2.7 | 0.0 | 2.4 |
| ophilily (%) | 89.4 | 94.5 | 90.2 | 84.2 | 84.5 | 88.1 | 87.2 |
| 95% CI | 84.8-94.0 | 89.8-99.2 | 84.7-95.7 | 78.8-89.6 | 78.6-90.3 | 83.4-92.8 | 82.1-92.3 |
| | | 0.17 | 0.84 | 0.15 | 0.19 | 0.70 | 0.53 |
| iced exophilily | - | NS | NS | NS | NS | NS | NS |
| OD FEEDING | | | | | | | |
| nd fed | 52 | 16 | 22 | 55 | 39 | 42 | 53 |
| nd fed (%) | 30.6 ^a | 17.6 ^b | 19.6 ^{ab} | 31.1 ^a | 26.4 ^a | 22.7 ^{ab} | 32.3 ^a |
| 95% CI | 23.7-37.5 | 9.8-25.4 | 12.3-27.0 | 24.3-37.9 | 19.3-33.5 | 16.7-28.7 | 25.2-39.5 |
| | | 0.02 | 0.04 | 0.92 | 0.40 | 0.1 | 0.7 |
| nd feed bition (%) | - | 42.5 | 35.8 | NS | NS | NS | NS |
| RTALITY | | | | | | | |
| rall mortality | 2 | 24 | 27 | 51 | 24 | 32 | 26 |
| rall mortality | 1.2 ^a | 26.4 ^{cd} | 24.1 ^{cd} | 28.8 ^d | 16.2 ^{bc} | 17.3 ^{bc} | 15.9 ^b |
| 95% CI | 0-2.8 | 17.3-35.4 | 16.2-32.0 | 22.1-35.5 | 10.3-22.2 | 11.9-22.8 | 10.3-21.4 |
| | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ected rol (%) for | - | 25.50 | 23.20 | 27.97 | 15.22 | 16.3 | 14.9 |
| 95% CI | - | 16.5-34.5 | 15.4-31.0 | 21.4-34.6 | 9.4-21.0 | 11.0-21.6 | 9.4-20.3 |
| tality of ds | 2 | 21 | 23 | 43 | 20 | 26 | 21 |
| of dead / l dead (%) | 100.0 | 87.5 | 85.2 | 84.3 | 83.3 | 81.3 | 80.8 |
| ed dead / total | 1.2 | 23.1 | 20.5 | 24.3 | 13.5 | 14.1 | 12.8 |

| | | | | | | | |
|-----------------------------|----------|----------|----------|-----------|----------|-----------|-----------|
| | | 0.00 | 0.00 | 0.00 | 0.0 | 0.00 | 0.00 |
| ected for rol (%) | - | 22.2 | 19.59 | 23.4 | 12.5 | 13.0 | 11.8 |
| mediate tality | 0 | 9 | 8 | 18 | 7 | 11 | 11 |
| o imm / total tality (%) | 0.0 | 37.5 | 29.6 | 35.3 | 29.2 | 34.4 | 42.3 |
| i mortality / l (%) | 0.0 | 9.9 | 7.1 | 10.2 | 4.7 | 5.9 | 6.7 |
| | | 0.00 | 0.00 | 0.00 | 0.0 | 0.0 | 0.0 |
| ected for rol (%) | - | 9.9 | 7.1 | 10.2 | 4.7 | 6.0 | 6.7 |

outcomes shaded in grey

Table 5. The mean knockdown and 24 h mortality induced by net pieces, cut from nets 'held back'

| Nets | Number washed | Mean | 95% Conf. Interval | | p-value |
|---------------|---------------|------|--------------------|--------|---------|
| | | | lower | upper | |
| Interceptor® | 0 | 76 | 59.76 | 92.24 | 0.0036 |
| SafeNet LLIN® | | 100 | 100 | 100 | |
| Interceptor® | 20 | 84 | 74.95 | 93.05 | 0.0225 |
| SafeNet LLIN® | | 96 | 89.97 | 102.03 | |
| Interceptor® | 0 | 76 | 59.76 | 92.24 | 0.0036 |
| SafeNet NF® | | 100 | 100 | 100 | |
| Interceptor® | 20 | 84 | 74.95 | 93.05 | 0.3823 |
| SafeNet NF® | | 90 | 77.84 | 102.16 | |

Table 6. The mean knockdown and 24 h mortality by hut-used and held back LLINs

| Net type | No. of washes | % Mortality | |
|-------------------|---------------|-------------|----------|
| | | Held Back | Hut Used |
| Interceptor® LLIN | Unwashed | 100.0 | 75.3 |
| | 20x washed | 50.0 | 83.5 |
| SafeNet NF® | Unwashed | 100.0 | 100.0 |
| | 20x washed | 75.0 | 89.7 |
| SafeNet® LLIN | Unwashed | 97.9 | 100.0 |
| | 20x washed | 77.1 | 95.9 |

Table 7. Results for the test systems' species and *Kdr* identification

| Test System | Species | | | <i>Kdr</i> | | |
|-------------------------------------|-------------------------|-----------------------|-------|------------|-----|-----|
| | <i>An. gambiae s.s.</i> | <i>An. arabiensis</i> | Other | RRe | RSe | SSe |
| <i>An. gambiae s.s.</i> Kisumu | 88 | 0 | 0 | 0 | 0 | 88 |
| <i>An. arabiensis</i> (wild caught) | 0 | 88 | 0 | 0 | 0 | 88 |

Figures

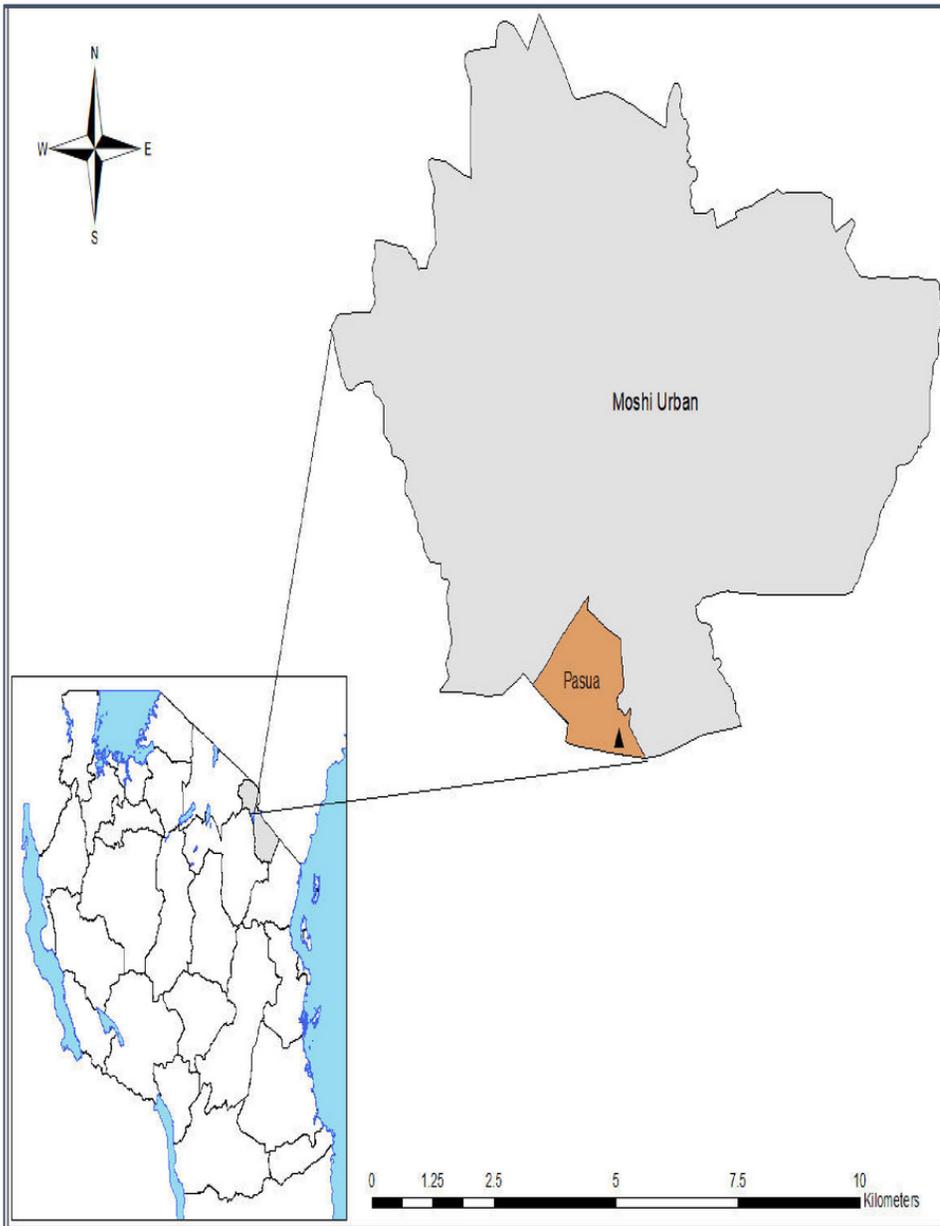


Figure 1

Map indicating the Pasua field station in Moshi, Northern-Eastern Tanzania, where the field evaluation of the LLINS was conducted.

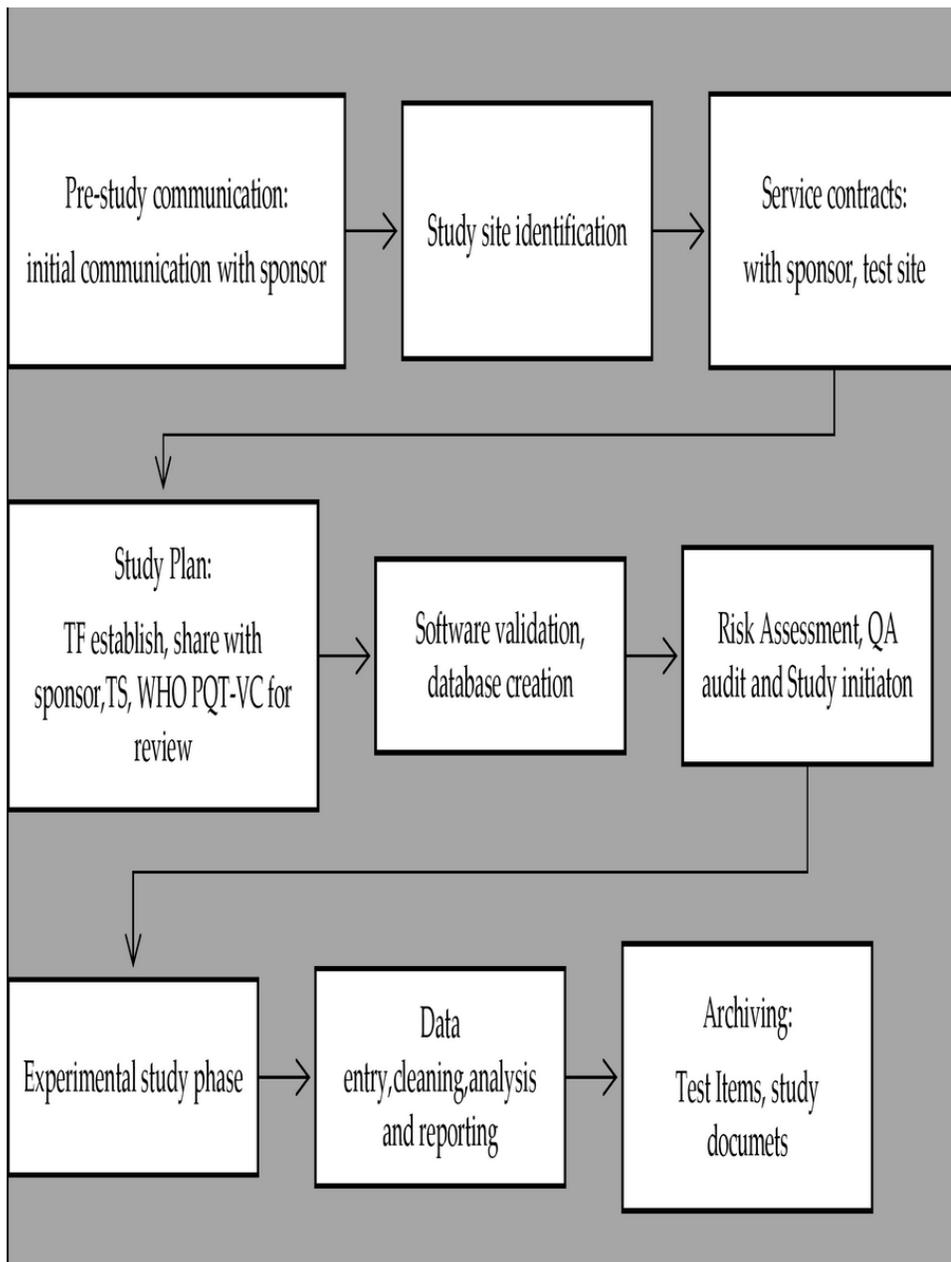


Figure 2

GLP study work flow. The boxes indicates the GLP procedures performed from the study initiation to the study completion

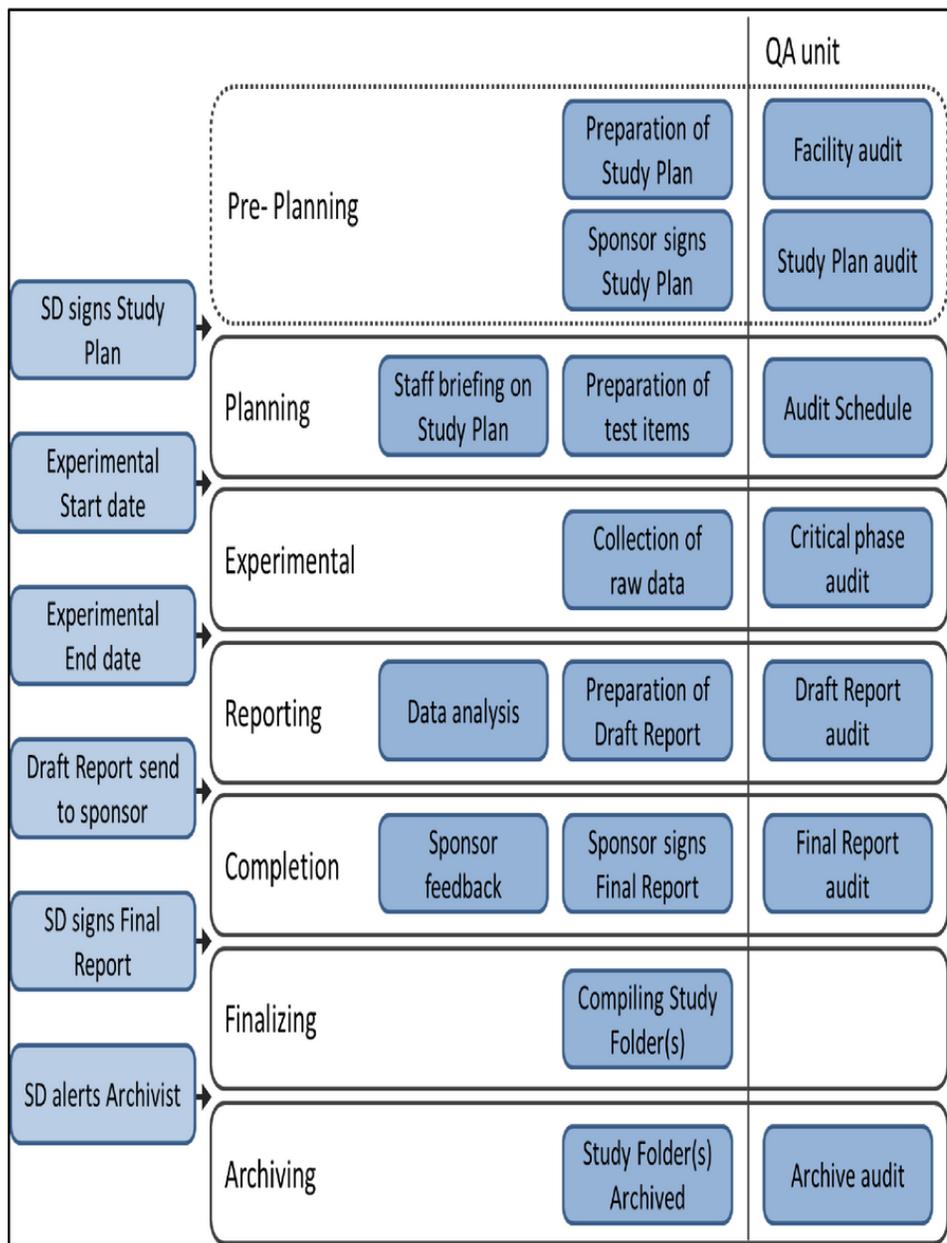


Figure 3

Phases for GLP study; from pre-planing to archiving phase

Nets 'held back'

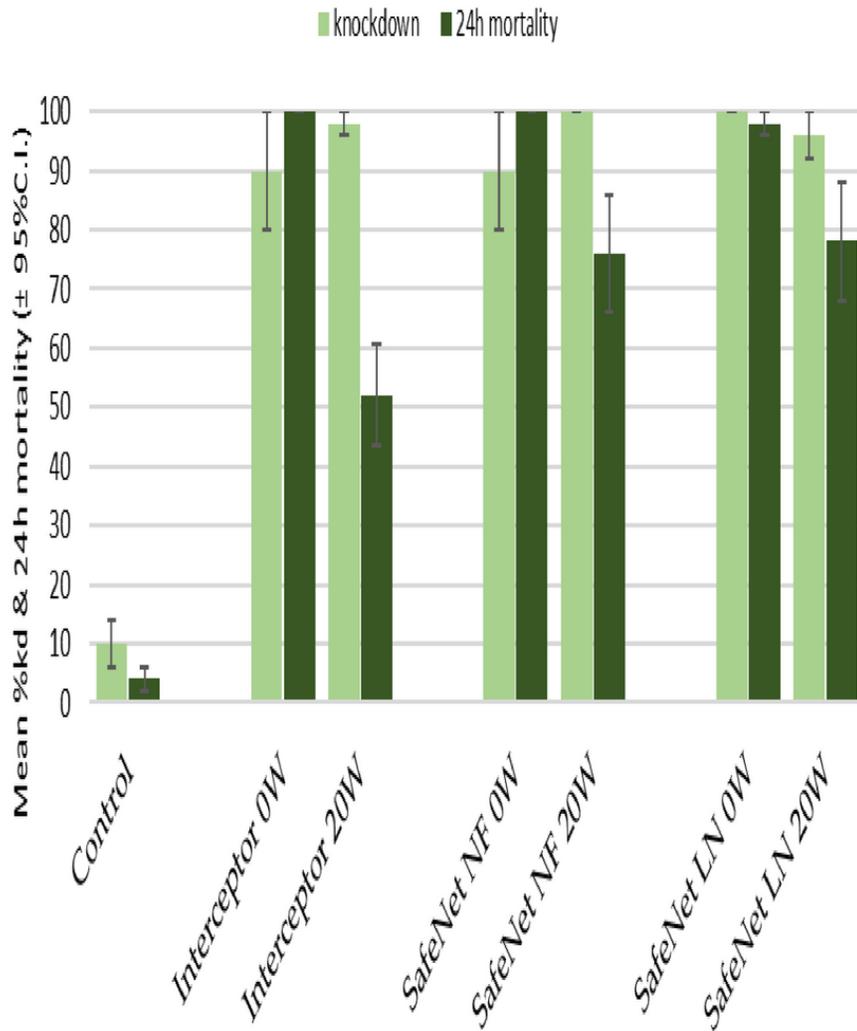


Figure 4

The mean 60 min knockdown and 24 h mortality for the *An. gambiae* Kisumu strain, after 3-minute exposure in cone assays against pieces cut from held back nets

Nets from experimental hut trial ('hut used')

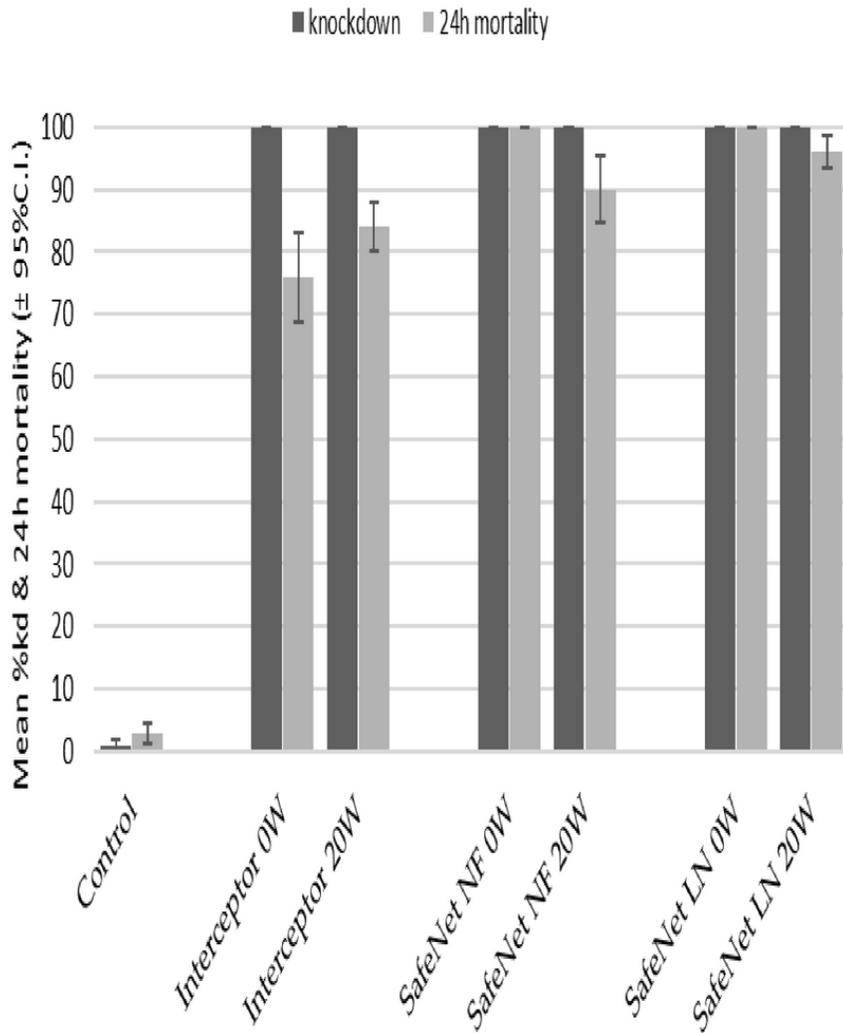


Figure 5

Mean percentage 60 min knockdown & mean 24 h percentage mortality for *An. gambiae* s.s. Kisumu strain, after 3-minute exposure in cone assays against pieces cut from hut-used nets.

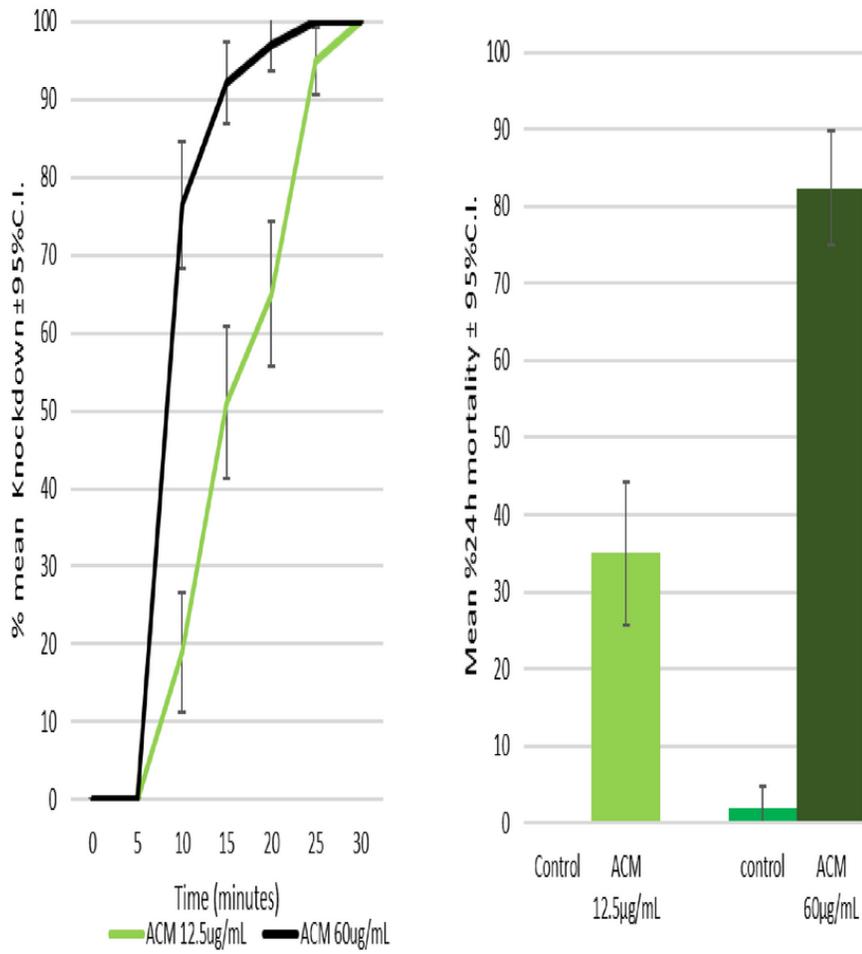


Figure 6

a) Mean percentage knockdown b) mean percentage mortality at 24 h, for *An. arabiensis* after 30 minute exposure to ACM at 12.5 µg/mL (1x diagnostic dose) or 60 µg/mL (5x diagnostic dose).

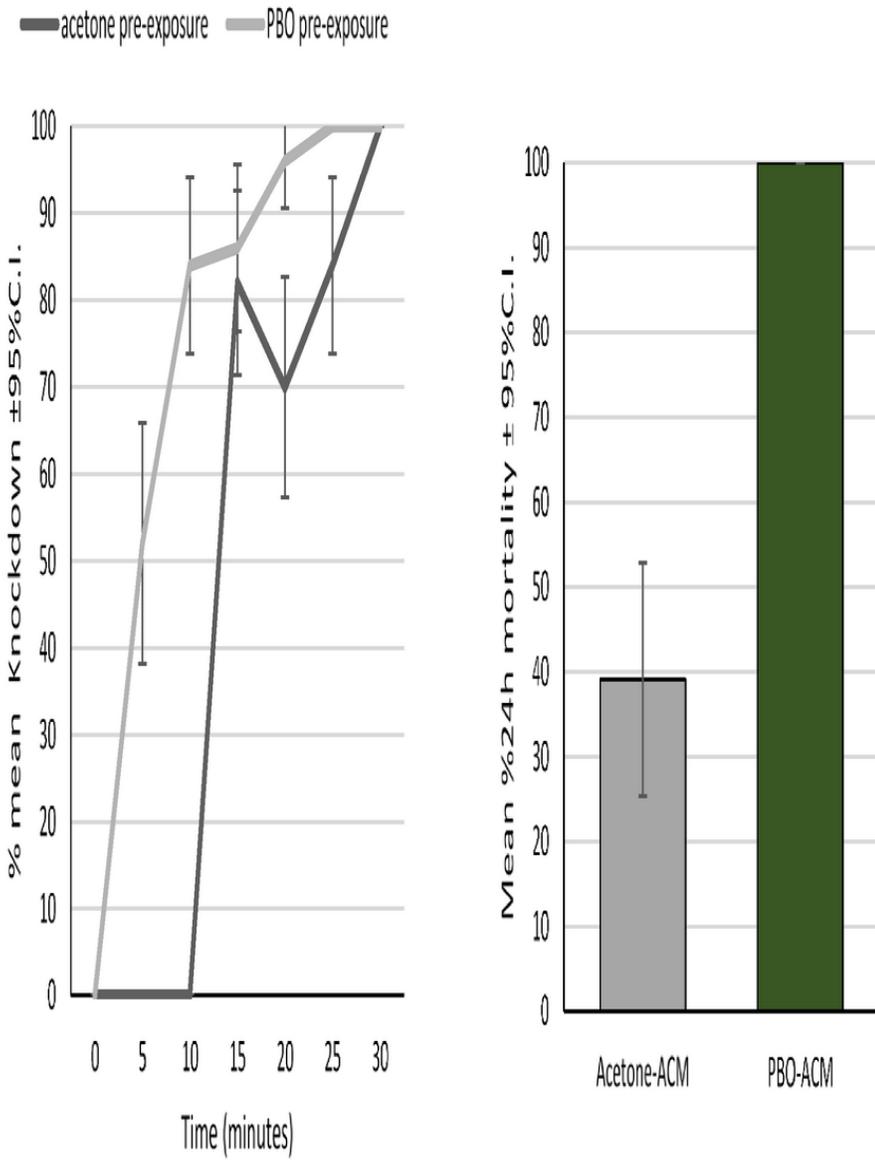


Figure 7

a) mean percentage knockdown & b) Control adjusted-mean percentage mortality at 24h, for *An. arabiensis* after 30-minute exposure to ACM at 12.5µg/mL, following a 1hr pre-exposure to acetone or PBO.

Body mass of test system
Anopheles gambiae s.s. Kisumu

Wing length of test system
Anopheles gambiae s.s. Kisumu

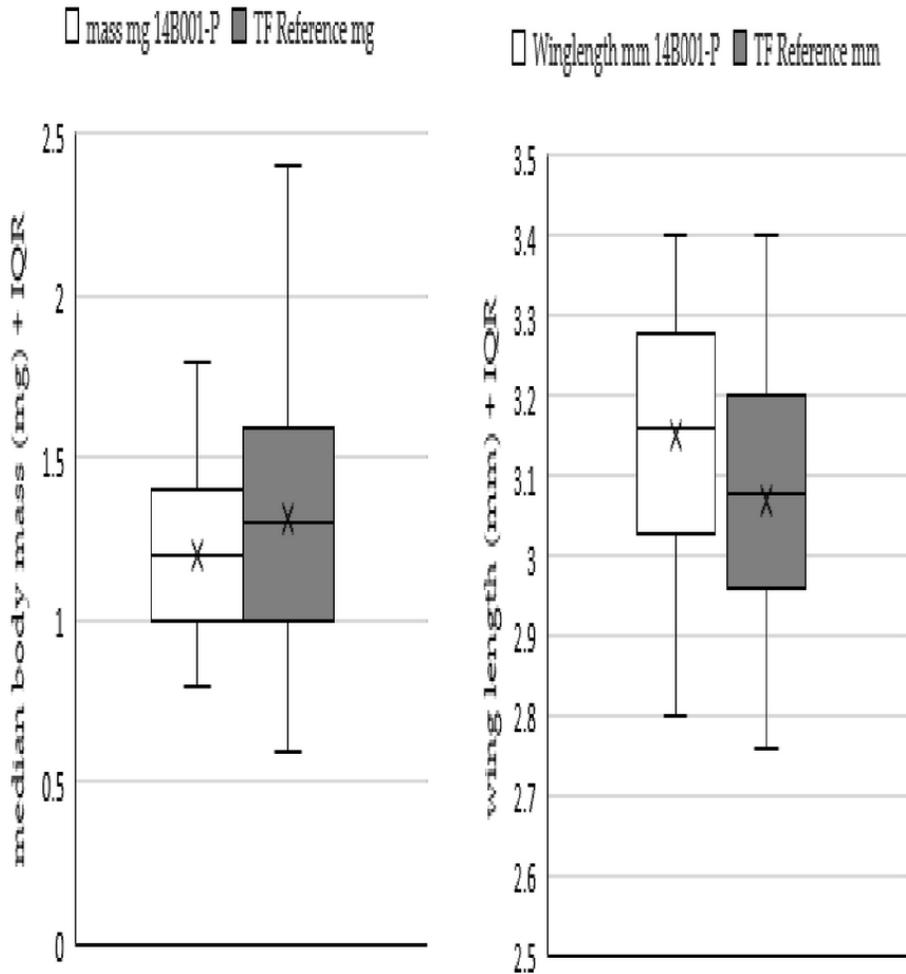


Figure 8

Box and whisker plots for (from left to right) mosquito body mass and wing length. Means are indicated by X. TF = Test Facility. IQR = Interquartile range.

Supplementary Files

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