Comparative evaluation of standard and dual-treated LLIN efficacy in Sud Ubangi, Democratic Republic of Congo (version 4.1)

Proposal/protocol

See Executive summary document for overview

Contacts:
David Weetman, LSTM david.weetman@lstmed.ac.uk
Seth Irish, CDC/PMI xjs7@cdc.gov

Project partners

LSTM (Janet Hemingway, David Weetman: project lead, responsible for study design, LSTM ethics, LLIN randomisation, molecular entomology, staff training at ANCs (with UKSPH), data collation and analysis
UKSPH: University of Kinshasa School of Public Health (Paul Mansiangi overall project management and liaison with PNLP (NMCP); recruitment of coordinators; organization of ANC training
Against Malaria Foundation (Peter Sherratt): LLIN donation and delivery to DRC; provision of additional LLINs to facilitate attrition testing
Global Fund (Marcos Patino Mayer): Supply of RDTs and drugs for ANC testing and treatment.
SANRU: distribution of RDTs and ACTs
IMA World Health, DRC: LLIN distribution in country to randomisation agreed with LSTM. GPS location of all houses and hanging of LLINs
CDC/ PMI-Vectorlinks/INRB (Seth Irish, Yung-Ting Bonnenfant, Richard Oxborough; Tiffany Clark; Ferdinand Ntoya, Fiacre Agossa, Francis Wat'senga): coordination and performance of entomology and resistance testing; staff training for entomology; chemical analysis of LLINs
Imperial College (Tom Churcher): data modelling

Project staff

Project coordinator (full-time, based in Gemena): epidemiological specialist to be recruited from University of Kinshasa School of Public Health
Project entomological coordinator (part-time, based in Gemena) to be recruited as part of Vectorlinks-INRB programme, in consultation with DRC project lead
INRB staff (overseen by Frances Wat'senga): entomology training and mosquito specimen processing
LSTM research assistant (50% time): project SOP assistance, molecular entomology
Health centre staff: ANC testing, administering treatments
Local assistants entomological collections

Introduction

Distribution of long-lasting insecticide impregnated nets (LLINs) has been the basis of malaria prevention in Africa, accounting for the majority of the reduction in disease prevalence seen over the last decade. LLINs work by protecting the individual who sleeps under the net, but crucially also protecting the community in which the nets are distributed by killing the mosquitoes that try and feed on individuals sleeping under the net. Pyrethroid resistance in *Anopheles* malaria vectors is now widespread across Africa and recent evidence from a large randomised control trial (RCT) in Tanzania showed that pyrethroid resistance is impacting the effectiveness of LLINs (Protopoff et al. 2018). Whilst nets remain in good condition, personal protective efficacy can persist because the LLIN still acts as a physical barrier to stop biting, but the community effect from mosquito mortality is lost where resistance frequency and level are high.

All ‘first-generation’ standard LLINs use pyrethroid insecticides, whereas ‘next-generation’ LLINs use either an insecticide synergist piperonyl butoxide (PBO) plus pyrethroid, a pyrethroid plus another insecticide, and LLINs using two non-pyrethroid insecticides are expected to reach the market soon. The basic cost of manufacturing these nets is higher than the first-generation nets, increasing costs of mass distribution campaigns if universal LLIN coverage is to be achieved. In addition, the volume of next-generation LLINs that can be manufactured to order may also be limited. How we optimize the distribution of nets in the most cost-effective format to maximize disease reduction in a post-pyrethroid resistance age now becomes a major question. While RCTs or annual community-based prevalence surveys are the gold standard for assessing effectiveness of LLIN campaigns, cost and local capacity preclude application of this approach widely across Africa. Simpler, yet robust operational measures are needed to allow evaluation of LLINs across a wider range of transmission settings. An attractive option for epidemiological assessment is to use prevalence rates in pregnant women attending Ante-Natal Clinics (ANCs). There is a growing body of published evidence alongside unpublished data from on-going work in Tanzania and Kenya and a review by van Eijk et al. (2015) showing a strong correlation ($r \approx 0.85$) between observed survey prevalence and prevalence in pregnancy. Recent work in DRC using an MSF-supported ante-natal clinic visitor RDT testing plan as a supplement produced promising results for using such data as an informative correlate of wider population prevalence (Hellewell et al. 2018).

Whilst there is growing evidence for the impact of dual (PBO) LLINs, virtually nothing is known about their impact on insecticide resistance, yet this is a crucial knowledge gap for longer term potential, and use in insecticide resistance management programmes. Thus, alongside epidemiological indicators, measurement of the insecticide resistance profile of local mosquito populations, efficacy of the LLINs thereon (especially as the nets age) and the selective force exerted by different LLIN types (which can be assessed effectively using diagnostic marker frequency changes) are key data which should be gathered.

The Democratic Republic of Congo, DRC, is among the highest burden malaria countries, with preventative malaria control reliant primarily on LLINs; IRS (spraying) campaigns have not been attempted to date beyond highly focal commercial activities. Many parts of the country have relatively weak local infrastructure and capacity and transport links are often problematic. The insecticide resistance status of DRC vectors has been very poorly documented, but information is beginning to come through from a series of sentinel sites (Wat’senga et al. 2018) and occasional focal studies (e.g. Lynd et al. 2018), and presents a general picture of widespread pyrethroid resistance in the major malaria vectors, of which An. gambiae ss. is dominant in most sites. Country-wide LLIN distributions, typically occur at the province-level (N=26) and are now targeted on a 3-year rolling basis. The LLIN distribution in Sud Ubangi province represents the first wave of a major DRC-wide LLIN distribution campaign starting in 2019.

Aim and approach

Here we aim to employ a fully-powered ANC-based approach to assess the comparative effectiveness of PBO and non-PBO nets, and thus the relative benefits of the former over the life cycle of the LLINs. The study also proposes a well-powered programme of molecular entomological surveillance to assess selective force on the mosquito population and how the entomological efficacy of LLINs changes over time. If the approach proves successful and cost effective, it may become a template for comparative evaluation of next generation nets. Results will be transparent to all partners. Donors will find the results useful in their immediate net choices. The extra cost of providing PBOs in Sud Ubangi alone is $650k, so this pilot study is timely generating an evidence base for future decision making before replicating mixed LLIN distributions across other provinces in DRC and elsewhere.

Background information

Scoping visit

Scoping visits to Sud Ubangi involving staff from PMI and LSTM took place in late April 2019 and served to identify contact networks, challenges likely to be faced in this relatively remote location, and establish the feasibility of the plans presented.

Sud Ubangi and LLINs: AMF have purchased approximately 1.9 million LLINs for Sud Ubangi province. LLINs are projected to arrive for distribution in November 2019 and will be distributed to the 16 health zones in Sud Ubangi (Figure 1). The 16 health zones (zones de santé) serve approximately 3.3 million people, in a total province area of approximately 52,000 km^2.

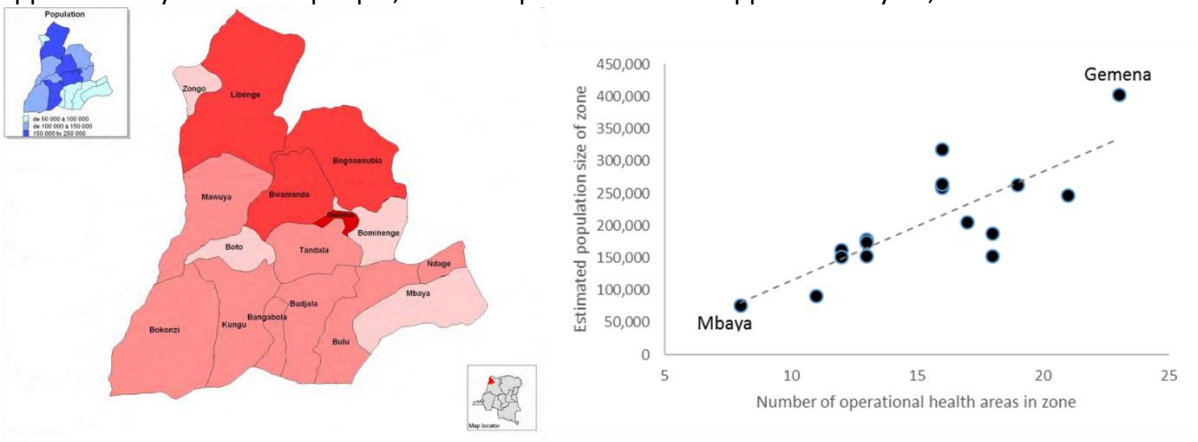


Figure 1. Map of Sud Ubangi health zones and the number of operational health areas per health zone in relation to population size

Population density is generally low, but is notably highest in the province capital Gemena, which has an airport served by twice weekly Congo Airways flights from Kinshasa. With the exception of the airport road in Gemena, paved roads are absent throughout the province, and degradation is likely to affect transport logistics, especially in the wetter months of the year (typically July-September). Health zones are divided into health areas (aires de santé), the numbers of which vary roughly in relation to population density (Figure 1). The boundaries of these health areas are not always precisely mapped, but each health zone's central hospital has a list of operational health areas, each of which is served by a health centre or (smaller) health post, the distances of which from each central hospital are recorded and hand-drawn maps are usually available locally. Socioeconomic status is generally low and housing near-ubiquitously of the traditional mud brick and thatch (photograph below).

Malarial epidemiology: Cases of malaria do not appear to be strongly seasonal in Sud Ubangi, although some slight decreases might occur during the drier months (December-February/March) (Figure 2). Testing rates are extremely high, barring a relatively short RDT stockout period in October-November 2018, and a very high and consistent percentage of suspected cases are confirmed by tests. Over 95% of infections are *Plasmodium falciparum* with few cases attributed to *P. ovale* and *P. malariae*. The number of recorded cases appears to have been increasing from the start of the DHIS2 recording period at the beginning of 2017. Whether this is a true reflection of prevalence, as a result of bednets beginning to lose integrity (Janko et al. 2018) after the 2016 distribution, or a result of improved data gathering is not clear.

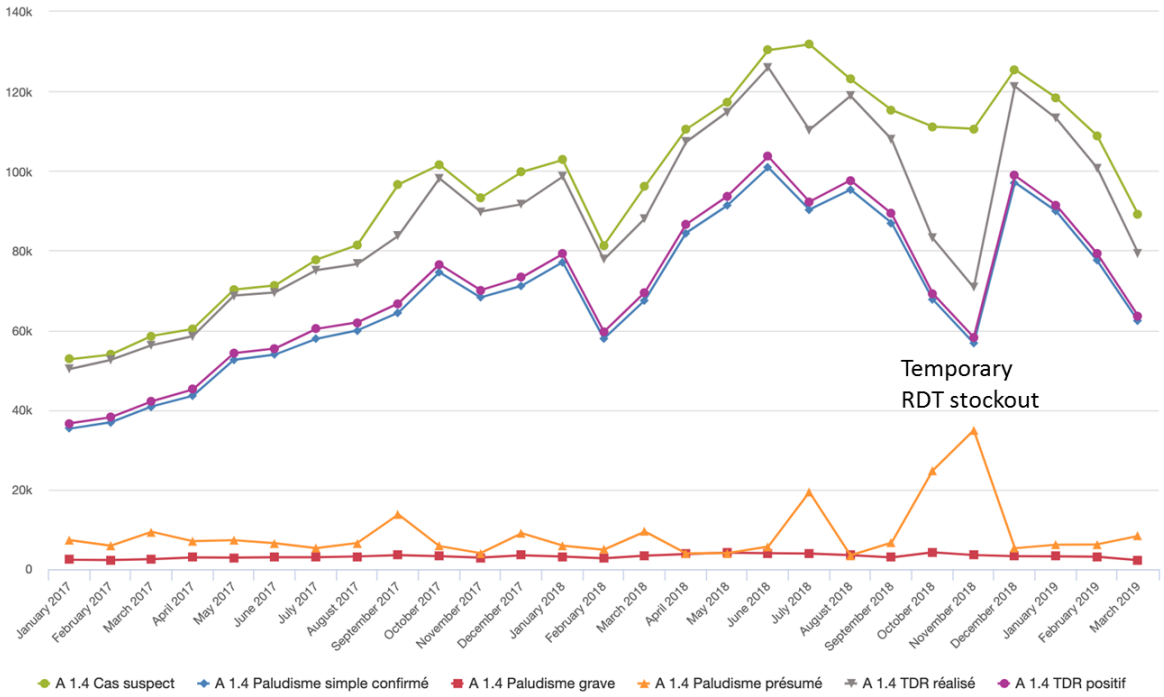


Figure 2. Malaria case data January 2017-March 2019. Testing rates (by RDT) are generally very high and the majority of suspected cases are confirmed as malaria; severe malaria is uncommon.

Malaria entomology and *Anopheles* insecticide resistance: There are over 60 *Anopheles* species recorded in DRC of which approximately ten are thought to be malaria vectors. There is no published or unpublished information on the anopheline fauna of Sud Ubangi but our previous work - for a baseline survey preceding an AMF-funded LLIN distribution in April 2016 - from neighbouring Nord Ubangi province is likely to provide a useful indicator (Lynd et al. 2018). Collections from villages in two of the health zones in Nord Ubangi detected 84% *Anopheles gambiae* s.s., and all larval collections in pools in or near the villages were this species. Moreover, recent collections of adults during our scoping visit in April 2019 from Karawa, on the border of Nord and Sud Ubangi were composed solely of *An. gambiae* s.s (N=279). Resistance in Nord Ubangi was prevalent to deltamethrin and especially permethrin (Figure 3), likely in part a result of the ubiquity of target site mutations: all individuals possessed *kdr* 1014 mutations (>90% either 1014F/F or 1014F/S) and wild type (1014L/L) susceptible alleles were absent, a pattern also found in the recent collections in Karawa. However, importantly PBO had a strong effect on mortality in tube bioassays, almost fully returning susceptibility for deltamethrin. Preliminary testing of LLINs (N=2 for Permanet 2; N=1 for other LLINs) detected a very poor performance of Permanet 2, with zero mortality (and <1% knockdown), but much better performance of Permanet 3, especially from exposure to the top panel, which contains both PBO and deltamethrin. Overall these results suggested that, unless there are substantial effects on mosquito fitness not captured within the 24h mortality assessment period, first generation LLINs are likely to serve primarily as a physical barrier with limited wider community effect. Comparability of results for Nord and Sud Ubangi is yet to be determined but, give their proximity, the similarity of results from Karawa on the border of the provinces, and environmental similarity, there are no *a priori* reasons to expect major differences between the provinces in mosquito communities.

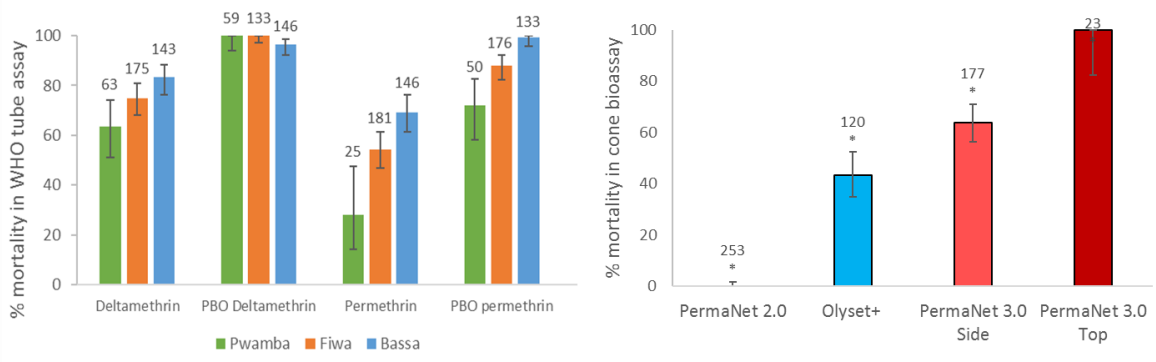


Figure 3. Insecticide resistance results from Nord Ubangi (March-May 2016). Left panel shows WHO tube bioassay results (60' exposure; 24h mortality) with the most common pyrethroids in LLINs, with and without PBO (left), tested on female *An. gambiae* from three villages. WHO cone bioassays on LLINs (3' exposure; 24h mortality); note that only the top surface of a Permanet 3.0 contains deltamethrin + PBO. For the cone assays, results should be regarded as indicative rather than conclusive because of the low number of tests. In both plots error bars are 95% confidence limits and labels show the number of females tested.

The cause of the substantial impact of PBO is difficult to determine exactly because of complexity in its actions, with capacity to act as an adjuvant (enhancing insecticide penetration) and to inhibit some esterase enzymes that may be involved in insecticide detoxification. Nevertheless, the primary mode of action is broad inhibition of P450s, the most important enzymes for pyrethroid metabolism (Snoeck et al. 2017). This suggests that enhanced activity of P450 enzymes is likely to be linked with the resistance profile shown in Nord Ubangi. Recent and ongoing work at LSTM, funded by NIAID and Wellcome Trust, has identified a candidate P450 gene region via a major selective 'footprint' in the *An. gambiae* genome, followed by finer scale investigation of polymorphisms therein and dynamics in field populations (unpublished data). Specifically, a haplotype (an extended allele encompassing a transposable element insertion, a gene duplication and a point substitution) has been identified in a cluster of Cyp6 P450 genes which has been rising rapidly in frequency at a rate of approximately 5% per year. The marker haplotype has recently reached fixation in East Africa; is increasing in frequency in DRC, and in Nord Ubangi was at a frequency of 61% in 2016, with genotyping of the recent Karawa collections showing a frequency around 10% higher. Importantly, possession of the marker provides strong protective efficacy against deltamethrin, whether assessed in tube bioassays (odds ratio = 2.2; CI 1.4-3.4) or against the sides of a Permanet 3 in a cone bioassays (odds ratio = 3.1; CI 1.9-5.1). In a population already in possession of very high frequency *kdr*-based resistance, addition of a strong metabolic mechanism, for which the haplotype serves as a marker (and probably contains the causal elements for the mechanism), provides a pathway to high intensity resistance. Markers are readily screened from dried specimens and provide an informative and tractable DNA diagnostic for metabolic resistance, appropriate for the Sud Ubangi *An. gambiae* population.

Data on mosquito abundance, infective *Plasmodium infection* rate, entomological inoculation rate (EIR), and human biting rates are all lacking for Sud Ubangi or any comparable province. Preliminary data obtained during our scoping study in late April 2019 via human landing catches gives some insight into biting patterns. The mosquito genera captured were *Culex* sp. (51%), *Mansonia* sp. (19%) and *Anopheles* species (30%), with similar total catches of the latter indoors and outdoors

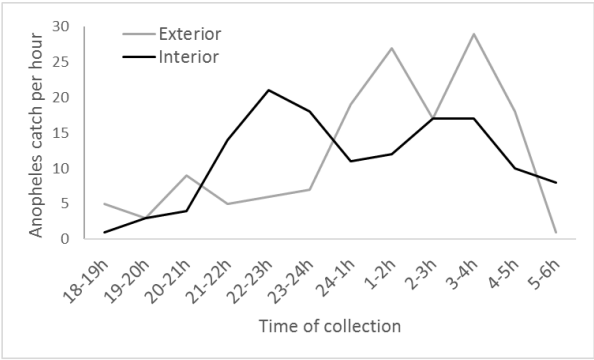


Figure 4. Human landing catch collections of *Anopheles* performed inside and outside five houses in a village near Karawa, Nord Ubangi (50 km east of Gemena) on 25/04/19

(Figure 4). Outdoor collections were close to houses, and do not provide evidence of an outdoor biting mosquito phenotype. Overall, these limited data suggest most biting occurring between about 9 pm and 5 am, a period during which younger children at least are likely to be sleeping and for much of which all residents may be protected by LLINs. Investigation of human behavior in relation to mosquito biting activity will be interesting to understand risk of exposure and the period during which LLINs are capable of being effective.

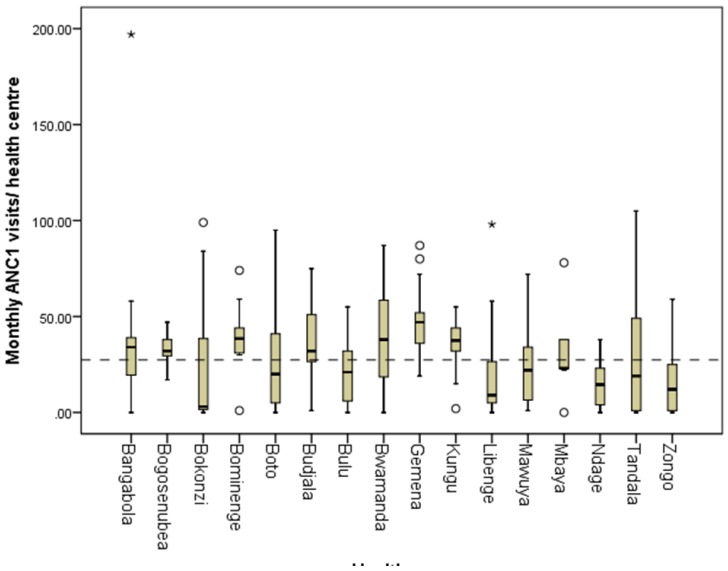


Figure 5. Monthly ANC visits for each health zone from DHIS2 data gathered from each health centre within aires de santé in 2017-18. Boxes show 25-75 percentile range, with median line indicated, and whiskers show 5-95% range. Circles are values for centres falling outside of the range and asterisks show statistical outliers. The overall average is shown by the dashed line.

Ante-natal clinic data: ANC data are available from DHIS2, and include number of first visits (ANC1) and symptomatic malaria cases and test rates in the women presenting. Prevalence rates are not available because there is no routine testing, only SP preventative distribution as per national policy. With the exception of an extreme outlier (top left in figure 7), there is reasonable consistency in the number of ANC1 visits per health centre per month across health zones. Notably, at least 7 health centres have at least 20 ANC1 visitors per month for every zone (and most many more) suggesting these can serve as useful *minimum* values for number of available health centres and ANC1 visitors for prevalence testing.

Randomisation and proposed LLIN-type distribution plan

Health zone	Net Need	Cumulative remainder non-PBO	Cumulative remainder PBO	Final assignment
Budjala	86,263	847,237	933,500	Non-PBO
Bwamanda	151,440	847,237	782,060	PB0
Bogosinubia	105,780	741,457	782,060	Non-PBO
Gemena	232,158	741,457	549,902	PB0
Libenge	146,629	594,828	549,902	Non-PBO
Mawuya	97,755	497,073	549,902	Non-PBO
Mbaya	40807	497,073	509,095	PB0
Tandala	177,066	497,073	332,029	PB0
Bulu	90,793	406,280	332,029	Non-PBO
Kungu	137,104	269,176	332,029	Non-PBO
Bangabola	119,323	269176	212,706	PB0
Bominenge	97,143	172,033	212,706	Non-PBO
Bokonzi	136,110	172,033	76,596	PB0
Ndage	85,008	87,025	76,596	Non-PBO
Boto	113,992	-26,967	76,596	87,025 Non-PBO + 26,967 PBO

Zongo	49607	-26,967	26,989	PB0

The proposed assignment uses the same adaptive randomization methodology used in the ongoing Uganda LLIN-UP trial. The process involves: (1) A value of 0 or 1 is randomly assigned to each cluster (zone), with assignment of the first cluster from a coin toss. (2) Cumulative probability ranges are generated for each net type based on the targeted number of each individual type of net / targeted number of total nets. (3) The second cluster is assigned to intervention-arm based on which cumulative probability range the corresponding random number fell into. (4) Steps 2 and 3 are repeated after removing the number of targeted nets assigned to the cluster for the corresponding type of net and the total number of nets. (5) The process is repeated until all clusters are allocated to an intervention.

The geography of the LLIN distribution plan is shown in Figure 6. Although during our scoping visit we were unable to obtain accurate information on travel times from Gemena to outer locations, our experience during recent visits suggests that travel times are unlikely to exceed an average rate of 30km/ hour and may be slower after heavy rains.

IMA world health will be organizing the logistics of the LLIN distribution including a per house hang-up programme involving GPS location of every house.

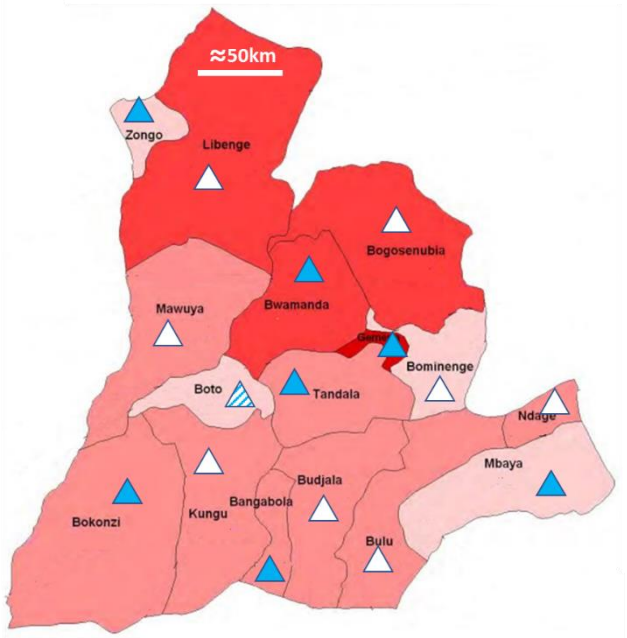


Figure 6. Map of Sud Ubangi health zones showing those randomised to receive PBO LLINs (blue); standard LLINs (white) or a mixed zone (blue/white)

Boto mixed LLIN distribution zone

There needs to be one mixed assignment zone (Boto), which will primarily be a non-PBO distribution (see Table). For reasons of study power (below) it is crucial to organise the distribution such that the net types cluster around health centres used for assessment, to ensure that the zone can be retained as a cluster for at least one and hopefully both LLIN types in the study. The number of available PBO LLINs represents just under 25% of the total net need for Boto. As a result of logistical considerations and also to reduce the potential for mosquito dispersal between clusters with different types of LLIN a distribution plan to a cluster of western aires de sante is proposed for Boto (Figure 7).

Figure 7. Map of Boto health zone showing the western areas of Bomele, Bombili, Bobandu and Bokozo proposed to receive PBO LLINs (blue); all other areas will received standard LLINs. ?-1,2 show uncertain boundary locations between zones

If projected LLIN needs for each of the four areas are accurate, this would leave 2,000 surplus PBO LLINs, which could be distributed as close as possible, i.e. preferably along borders of these aires de sante to expand the size of the PBO-LLIN cluster.

More generally the mixed distribution in Boto could provide an important template for future distributions to provinces where division into fewer zones would preclude a statistically-useful comparative study if LLINs of contrasting types were distributed solely at the zonal level. Whilst statistically a distribution fully randomized at the level of aires de sante would be powerful, this is logistically challenging, may lack visible mapped impact on a malaria data map. Grouping of aires de sante - in the way proposed for Boto - could provide a viable intermediate distribution plan to meet statistical requirements, and, in addition to relative logistical ease also has the advantage of reducing the impact of human, net and mosquito movements among net-type clusters.

Proposal hypotheses and endpoints

Epidemiological

Primary
Hypothesis: PBO LLIN distribution will result in a lower malaria prevalence than standard (non-PBO) LLINs as indicated by reduced prevalence in ANC1 visitors.
Endpoint: monthly malaria prevalence in ANC1 visitors measured using RDTs, with main assessment points at 6 monthly intervals and intermediate assessments quarterly.

Sample size calculation and power: From preliminary analyses it was evident that a minimum 8 vs 8 cluster/ arm trial would be required to adequately power the study to detect plausible effect sizes using a relatively simple cluster arm comparison for a single month's data (temporal variation is unknown so it is difficult to include multiple months in *a priori* power analysis). We used fixed assumptions for the number of available health centres/ zone of N=7 and number of ANC1 visitors

per month of N=20, giving a cluster size of N=140. Note that varying the cluster size has only a moderate impact on power, provided health centres are chosen randomly from those available. Parameter values for the inter-cluster coefficient of variation (*k*, which has a large impact on power) were estimated from DHIS2 2018 data from the rate of positive malaria tests per month per capita (i.e. data divided by population size of zone) as a proxy. Following equation 9 in Hayes & Bennett (1999) we calculated k=0.18 from these data. However, noting this is lower than estimated from the only comparable published study from Tanzania (k values of 0.20-0.28 depending on survey round; West et al. 2014) we also examined a 'high COV' value of k=0.3.
Similarly we lacked information on the baseline prevalence of malaria for Sud Ubangi, with the last survey in 2013-14 detecting a rate of 24% in children under 5 (MPSMRM 2013-14), which using a conversion factor of 1.44 between child and pregnant women's prevalences (van Eijk et al. 2015) we used to estimate an ANC prevalence value of 17%, which is also very close to the average figure for the DRC ANCs presented in Hellewell et al. (2018). However, figures in Hellewell vary greatly and thus because of the considerable uncertainty in this point estimate, we examined a range of possible prevalence rates (applicable to the standard-LLIN reference arm).

We identified the minimum detectable reduction in prevalence using equation 4 from Hayes & Bennett (1999) with the following study design parameters:
Arms=2; Clusters (zones)/arm = 8; subjects/ cluster = 140; k = 0.11 or 0.30; α=0.05; power =80%; prevalence in reference standard-LLIN arm (π0) varied between 5 and 50%

Results are shown in Figure 8, and suggest that the study design proposed will be capable of detecting a reduction in prevalence of less than the maximum obtained in the Tanzania Olyset + PBO LLIN trial (Protopopoff et al. 2018) even with the higher COV unless prevalence rates are much lower than expected and if the lower COV value is realistic the study has good prospect to detect the lower effect size seen in the Tanzania study with likely prevalence rates. Our data from Nord Ubangi suggest a greater entomological efficacy of Permanet 3 than Olyset + (Figure 3) but it is unclear whether this might translate into a larger epidemiological effect size than obtained in the Tanzania Olyset+ trial. The split zone in Boto could provide one additional non-PBO cluster, if the distribution occurs as planned and can include sufficient health centres, but at present the sample size calculations and power analysis conservatively retain the 8 vs 8 design.

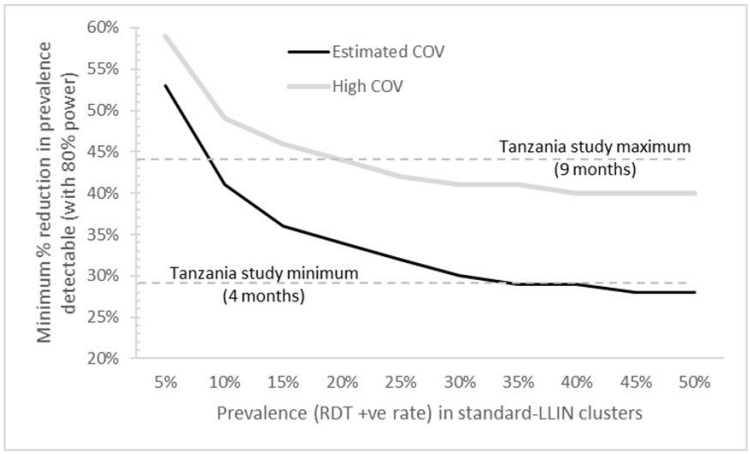


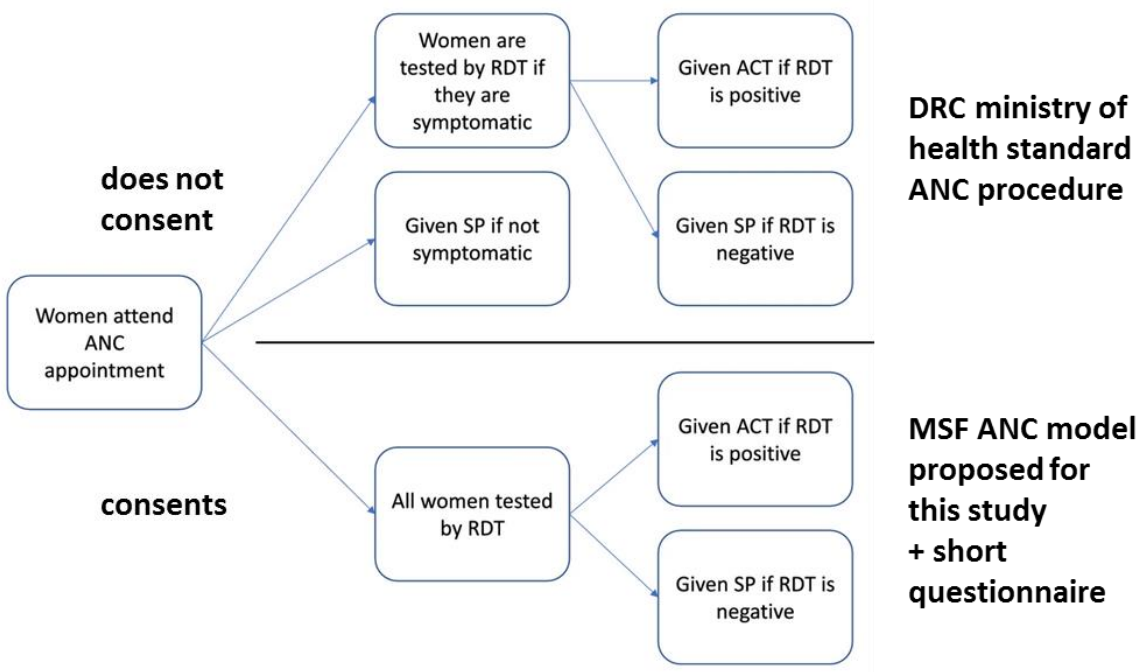
Figure 8. Summary of power for ANC1 prevalence study based on sample size projections of 8 health zones/ arm, 7 health centres/ zone, 20 ANC1 visitors tested per month for two different values of the inter-cluster coefficient of variation. The plot shows the minimum % reduction in monthly prevalence in PBO vs non-PBO LLIN arms predicted to be detectable (with 80% power) as a function of *Plasmodium* prevalence in ANC1 visitors. Maximum and minimum % reduction estimates obtained in the Tanzania PBO (olyset +) vs standard (olyset) LLIN trial (Protopopoff et al. 2018) are superimposed for context.

Procedures

ANC visitor recruitment

The recruitment process at each clinic will occur when women attend their first (second trimester) ANC appointment. Women will be asked if they would like to be enrolled in the study, the main

implication of which for them is shown in the flow diagram below, i.e. they will be tested for malaria whether symptomatic or not. It will be made clear that there is no accepted benefit of either, and that the option is being offered solely for the purposes of our LLIN evaluation study. If, after asking any questions and discussing with friends or relatives as desired, they agree and sign the consent form, they will proceed via the lower route shown in the figure. If they do not wish to be included they will follow the route shown in the upper part of the diagram, which represents standard care. The decision will need to be taken within the timeframe of the appointment, and if a clear and certain decision is not reached by this time the standard care route will be followed.



Inclusion of health centres (aires de sante) in the study
The aires de sante (N=7) from each zone de sante (N=16) to be included in the study will be identified by random allocation from a list of functional aires de sante in a zone with >20 ANC1 visitors per month based on 2018 DHIS2 data.

Data collection and collation
Data collection should start at the first possible ANC before or just after (determined by RDT/ACT supply and training logistics) LLIN distribution. Recordings preceding the distribution would be beneficial but are unlikely to be feasible owing to time constraints.
It is proposed that study RDT results are recorded separately on the questionnaire form (appended) and transferred to the ANC register (observations section), rather than as a suspected or tested case in the standard register, which is the current procedure. The simple questionnaire will collect only essential information about the participant and their home environment as pertains to malaria risk and should be readily completed within the time waiting for the RDT result. Variables generated from the questionnaire data may serve as covariates in analyses although this is not planned *a priori*. ACT treatment would be administered to women testing positive in the RDTs. Data collected will be transferred monthly to Gemena in the same way as the DHIS2 data collation process, and passed to the study epidemiological coordinator.

Training
Nurses at health centres would be responsible for this additional testing and treatment and for the enrolment of participants into the study. Training in administering RDTs and ACTs according to the protocol and filling the register and questionnaires will be organized by the DRC project coordinator

and facilitated by the Gemena-based epidemiological coordinator, in coordination with a representative of the PNLP. It is planned that the training will be conducted in five regional pools to optimize cost and logistical demands. One member of nursing staff per health centre included in the study will be formally trained, along with a representative from each health zone.

Secondary

(i.e. to be tested between PBO and non-PBO areas but study not specifically powered for these)
RDT-confirmed monthly malaria cases and monthly severe malaria cases *per capita*. These data will be taken from DHIS2 which is updated monthly for all health centres in relation to population size estimates for each health zone. As noted earlier severe cases are quite rare with approximately 1/20 of the 83,150 malaria cases reported (and confirmed by RDT) in 2018 in the province being recorded as severe.

Epidemiology design summary: 8 zones per LLIN-type arm; 7 health centres per zone; 20 (or more) ANC1 women tested per health centre per month. *Provided the effect size is of the order seen in the Tanzania PBO trial, the design should be robust to variation in currently unknown parameters.*

Data analysis

All data will be analysed primarily on the basis of intention-to-treat. The primary outcome will be the prevalence of parasitaemia from the ANC visitor surveys, which will be compared between treatment arms using generalized linear Poisson mixed models with log link function to allow assessment of intra-cluster correlation, and correction factor to maintain type I error rate with a small number of clusters, but maintain power for the number of clusters in our study (Leyrat et al. 2017). Continuous (monthly) data will be available for each major endpoint analysis and will be analysed with time since LLIN distribution as a covariate. However, the power analysis above is based on separate monthly assessments. Interim endpoint analyses will be conducted quarterly, to allow ongoing assessment of the trial by funders. Meaningful baseline data will not be available for the study and therefore it is not intended to perform adjusted analyses *a priori*, though if systematic differences in influencing factors are identified, covariates may be incorporated into analyses. Blinding at the level of data collection is not possible because of the ready identifiability of the different types of LLINs. However, data source will be encoded prior to statistical endpoint analysis to ensure blinding.

Entomological

Primary

Hypothesis: PBO LLIN distribution will reduce the benefit of P450 metabolic-based enzymes and arrest selection on a key genomic area for resistance leading to lower frequencies in PBO-LLIN zones (via stasis or a fall) than observed in zones with standard LLINs (in which an increase is expected). In lay terms this is testing a hypothesis that PBO LLINs will reverse the increase in pyrethroid resistance; an effect which we should be able to capture using informative DNA markers for molecular surveillance.
Endpoint: comparative annual resistance marker frequency in *An. gambiae* measured using molecular surveillance diagnostics

Sample size calculation and power:

Entomological indices can be readily assessed at intervals with a periodicity of 6 months suggested as providing a good timeframe for a hypothesised change in marker frequencies. The work is

powered however for a 12-month comparison which it is anticipated would come from comparisons of collections made initially at 0 vs 12 months, but with additional comparisons (subject to study continuation) at 6 vs 18 months etc. We base calculations on a current base resistance marker frequency (the Cyp6 gene-cluster marker haplotype described above) of 0.72, which is based on recent local data we have obtained. A projected 5% annual decrease in the PBO arm and 5% increase in the standard LLIN arm, translates into a projected difference between arms of approximately 10% per year. The total number of *An. gambiae* required per collection period to detect such a difference as significant (using an exact test) is N=1392 (allowing a two-fold higher collections in standard-LLIN houses than PBO-LLIN houses). Clustering is not accounted for in the analysis because we postulate (based on general patterns for the species) that it is unlikely there will be significant *a priori* population genetic structure within the *An. gambiae* population of Sud Ubangi.

Allowing for 16% non-*An. gambiae* in collections (based on our data from neighbouring Nord Ubangi province; Lynd et al. 2018) equates to a total of 1,650 Anopheles per survey period. Allowing for an average catch of N=3 *An. gambiae* per standard-LLIN house, and 1.5 in PBO LLIN houses this would require a total number of N=734 houses to be sampled by prokopack collections during each collection around the 6 month periods. We will sample from 4 clusters per arm to reduce travel costs and time). Specifically, we will sample from 3 separate health zones per arm and then from both PBO- and standard-LLIN clusters within the mixed Boto health zone. Villages to be sampled will be those in close proximity to the health centres included in the epidemiological component of the study.

It is intended that the same houses would be sampled each time, though if this is not possible, or if verbal consent to access to sample is not provided a neighbouring replacement house would be recruited. The projected 10% decrease is annual and therefore the main comparisons would compare surveys separated by 12 months, i.e. months 0 vs 12, 6 vs 18, 12 vs 24, etc.

Secondary entomological outcomes

For the secondary entomological outcome, the density of malaria vector mosquitoes per house, the number of houses to be sampled to provide similar power is much higher, owing to the very high variability expected between houses (based on data from Protopopoff et al. 2018). Therefore, we will pool collections from the 6th and 12th month collection periods (providing a total of N=1468 house collections) to provide adequate power for this analysis to detect a two-fold difference in female *Anopheles gambiae* abundance between study arms (assuming a standard LLIN density of N=3/house).

Power calculations for or other main secondary entomological variable, the entomological inoculation rate, are prone to even higher variance but based on figures from (Protopopoff et al. 2018), the same strategy of pooled assessment from the 6 and 12 month collections as for density assessment should permit detection of the fourfold difference they observed (based on a standard LLIN mean of 1 and coefficient of variation of 6 in each arm).

Logistics: The poor transport network places limitations on feasibility of entomological collections in all zones and we therefore will sample from 4 clusters per arm, choosing those within closer travel distances from Gemena. The collection method proposed is by timed prokopak aspiration (10 min/house), providing a standardised and relatively inexpensive methodology for collecting adult mosquitoes, which can be used for abundance monitoring and infection assessment. In a large study evaluating LLINs in Uganda, aspirator collections have been made in houses (Lynd et al. 2019). Each zone involved will have a collection kit (prokopak, solar charger, batteries, etc) which will be transferred in turn to each of the four collection locations, within each of which we estimate approximately 25 house collections will be required, assuming catch rates of 3/house/collection in standard LLIN zones and half this in PBO LLIN zones (the sample size calculation allows for this imbalance). Collections should take approximately 6 weeks at the beginning of each 6-monthly phase of the study. Collections will be overseen by an entomological coordinator and performed locally by a health worker seconded from a health centre, assisted by a local facilitator in each villageMosquito collections from prokopaks would be transferred from aspirator cups into cages to allow removal of debris and non-target species and then aspirated into Falcon tubes with cotton wool over silica (1/house). Samples would be returned to Gemena for logging and basic sorting and then transferred to INRB Kinshasa for further processing, to include morphological species identification and detection of *Plasmodium* infection (sporozoite rate), before transfer of specimens to LSTM for molecular species identification and detection of *Plasmodium* infection (sporozoite rate), molecular diagnostic marker screening.

Prokopak collection in Tanzania; AIRS 2016

Training

In contrast to the epidemiological training, the entomological assistants will all be trained centrally at Gemena by INRB and Vectorlinks staff to permit the more in-depth training required.

Data analysis

The primary entomological outcome measure will be the frequency of the molecular deltamethrin resistance marker haplotype. A single species (*An. gambiae*) is predominant in the study area. *Anopheles gambiae* typically show limited genetic differentiation even over large spatial scales (Miles et al 2017) and it unlikely that initial marker frequencies will show spatial variation, as noted above. Therefore analysis is planned for an inter-arm comparison using an exact test procedure. However, we will also examine whether spatial autocorrelation is present and introduce a spatial component into analysis using a generalised linear model if this is the case. Other important entomological outcomes (below) include the abundance of mosquitoes. The first secondary entomological outcome – abundance of female malaria vector mosquitoes, which is likely to show much higher variance even at local scales will be analysed using a Poisson generalised linear model with negative binomial link function to determine whether a difference in the density per house between study arms is present annually (i.e. from two 6 monthly collections pooled). Similar analysis will be performed for a measure of the malaria transmission rate – the entomological inoculation rate, which is estimated as the mean number of sporozoite-infected mosquitoes per house per night, adjusted for the number of sleepers per house (identified from household questionnaire data), again based on the two 6 monthly collections pooled.

Key secondary entomological indicators (all to be measured biannually from the same collections)

Abundance of female Anopheles malaria-vector mosquitoes

Counted from the prokopak collections. Power calculations from the study design above suggest that detection of an annual 50% decrease in *Anopheles* abundance can be detected with >80% power.

Blood feeding rate of female Anopheles malaria-vector mosquitoes

From assessment of the bloodfed status of the above mosquitoes, coupled with limited random molecular testing of mosquito blood meals to quantify the human bloodmeal index.

Sporozoite rate in female Anopheles malaria-vector mosquitoes

Assessed from the same mosquitoes using molecular analysis of the head and thoraces of the females to determine the proportion infective.

Entomological inoculation rate

The number of infective bites/person/night calculated from preceding parameters and the number of sleepers in the house (obtained from questionnaire)

Pyrethroid phenotypic resistance prevalence and intensity (and PBO effects thereon)

Diagnostic dose bioassays are widely used but typically insensitive measures of insecticide resistance, but can be improved by addition of further assays using higher concentrations of insecticide to segregate more highly resistant females (CDC 2016; WHO 2017). A key component of the testing procedure is the use of PBO as a pre- or co-exposure pyrethroids to determine any evidence of loss of efficacy of PBO. We propose to test 3 different concentrations (+ no insecticide controls) of deltamethrin with and without PBO, using standard WHO tube test procedures on representative mosquitoes collected from two separate PBO and non-PBO zones (Figure 8). This yields a total of 2 arms x 2 collection locations x 4 concentrations x PBO+/- = 32 treatments, each of which will be represented by 4 replicate tubes of N=25 3-5 day old female *An. gambiae* s.l. The plan is shown below.

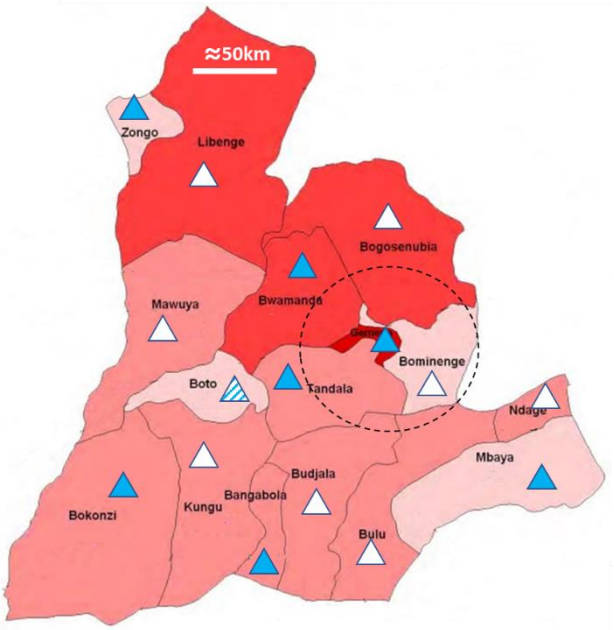


Figure 9. Health zones and distribution with feasible daily travel for mosquito collection and return to Gemena indicated by the dashed circle. It is planned that bloodfed females will be collected from locations within this zone and their offspring used for phenotypic assays of resistance and LLIN bioefficacy

- Deltamethrin 1x (diagnostic dose)
- Deltamethrin 5x
- Deltamethrin 10x
- Deltamethrin 1x with PBO pre-exposure (if 1x does not kill 100%)
- Deltamethrin 5x with PBO pre-exposure (if 5x does not kill 100%)
- Deltamethrin 10x with PBO pre-exposure (if 10x does not kill 100%)

The large number of mosquitoes required for testing at each period (6 monthly) suggests that a flexible approach is necessitated a priori until availability of different collection sources can be assessed during initial entomological surveys (above). There are two possibilities: collection of larvae (and rearing to adults), or collection of blood-fed females using more exhaustive prokopak collections and testing of their F1 offspring. Each methodology has logistical pros and cons but results are expected to be broadly comparable, provided the number of larval habitats sampled or egg batches raised is large enough to give a fair representation of the local mosquito population.

In addition, if sufficient females are available, additional insecticides can be tested on a more limited scale, for example the other major LLIN pyrethroids permethrin and perhaps alpha-cypermethrin or perhaps newer LLIN insecticides such as chlorfenapyr, which if samples of BASF Interceptor G2 were available might be best tested directly in assays on Sud Ubangi mosquitoes.

Net efficacy and lifespan

We need to know when nets start to lose integrity, so that a reduction of impact due to loss of coverage can be distinguished from a reduction of impact due to loss of insecticide. A simple durability monitoring protocol will record holes (and proportionate hole index calculated), coupled with bioassays on the nets, as well as chemical tests done to assess the quantity of deltamethrin and PBO present on the nets. Specifically, bioefficacy will be measured via WHO cone tests using locally-collected mosquitoes (see Figure 9 for collection areas) on sections of LLINs (with separate tests for upper and side panels for Permanet 3) removed from the field (and replaced) at time intervals of 6 months. The LLINs to be removed can be selected randomly from the houses identified for entomological collections above at the end of the mosquito sampling period. Sections of the same LLINs will be tested for active ingredient concentration (with separate tests for upper and side panels for Permanet 3) using HPLC analysis at LSTM. Per collection period we plan to remove an LLIN from a random selection of 10 houses from villages around health centres from 3 zones/arm, giving a total of N=30 LLINs per arm per time point. The 30 LLINs per arm will be tested using one bioassay cone per net for PermaNet 2 (standard) and two for PermaNet 3 (top and side) yielding a total required number of female mosquitoes for testing of N=900. Collections for these would be made at the same time as for insecticide susceptibility tests.

Household recruitment

Mosquito collections

Following initial contact between the head of each village to be included, randomly identified households (from a GPS list collected during planning for the LLIN distribution by IMA the distribution coordinators) within villages will be visited by study personnel, accompanied by a village resident. If householders are present, the purpose of the study and nature of collections will be explained to them, and if they are willing to be enrolled the head of the household will be asked to sign a consent form the following day, which will give potential permission (subject to verbal consent at the time of collection) to collect from the house on up to 6 occasions at 6 month intervals. If they do not wish to take part, or the head of the household is absent, a neighbouring house will be approached. Owing to the possibility that verbal consents will not be given, or householders may not be present in the property at the time of the collections (between 6am and 10am) we aim to consent

approximately 30% more households than will be sampled, and will call to inform those not included as soon as possible on the collection morning.

LLIN durability

Householder recruitment for quantification of LLIN integrity and removal sampling of LLINs will proceed in the same way as for mosquito collections, albeit on a much smaller scale and involving only a single visit per house. The houses will be different to those included in the mosquito collections and a separate information sheet and consent form will be used.

Additional work of interest but not currently planned or costed for:

Human behavior and malaria risk (highest priority)

The capacity of LLINs to protect is determined by human and mosquito behavior (timing and location of biting). The former can be assessed using simple questionnaires to determine location and sleeping times, and the latter is best assessed using human landing catches (HLCs) conducted indoors and outdoors, recording biting times of mosquitoes (Figure 4), which is quite expensive. If complementary funding became available it would be extremely informative to include this component in the study

Age distribution of the mosquitoes

Successful interventions targeting adult mosquitoes are expected to shift the age distribution of adult females downwards, which is an important component of their success because it reduces the relative proportion of females that have lived long enough for infecting *Plasmodium* to pass through their incubation period and become infective sporozoites. Unfortunately, mosquito age is difficult to assess, and currently most methods lack accuracy and/or are extremely time-consuming. However, methods based an expanded gene expression panel are currently under evaluation (LSTM, MRC Gambia collaboration), as are other methods for molecular analysis including mid-infra red spectroscopy (developed at University of Glasgow, UK). We will preserve a portion of samples in a way suitable for these kind of analyses (in RNALater preservative for gene expression or dried for MIR spectroscopy).

Molecular surveillance of additional local resistance mechanisms and broader genomic/transcriptomic changes

Samples gathered for DNA analysis (dried specimens) or for gene expression analysis (in RNALater) can also be used for comparative analysis of additional mechanisms of insecticide resistance (i.e. beyond *kdr* and the Cy6P haplotype markers) as they become available from ongoing work at LSTM.

Molecular surveillance of Plasmodium falciparum strain identity and diversity

Mosquito samples testing positive for infection can also be used as a source of *P. falciparum* to investigate genetic diversity (strain variation) using a molecular barcoding approach to type different strains.

Key deliverables

1. Epidemiological assessment of the efficacy of conventional and PBO-treated LLINs using ANC prevalence data
2. Assessment of the selective impact of different LLIN types on the local mosquito populations targeted using molecular entomological surveillance

3. Assessment of the impact of LLIN type on cases of uncomplicated and severe malaria as recorded in DHIS2
4. Assessment of the impact of different LLIN types on the local mosquito abundance, sporozoite rates and entomological inoculation rate
5. Assessment of the lifespan of different LLIN types from entomological assessment of bioefficacy, chemical analysis of active ingredients, and measurement of the physical integrity of a sub-sample of LLINs
6. Assessment of the effect of LLINs on phenotypic variation in resistance over time, including to the synergist PBO
7. Capacity strengthening of local health service especially in entomological surveillance

Costs and timeline

See document Study costs and timeline v2.xlsx

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