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Bio-efficacy evaluation of long-lasting insecticidal nets after five years of storage: implications for malaria control programm

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BIO-EFFICACY EVALUATION OF LONG-LASTING INSECTICIDAL NETS AFTER FIVE YEARS OF STORAGE: IMPLICATIONS FOR MALARIA CONTROL PROGRAMMES

Jeremiah John Musa

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Public Health Research of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Long Lasting Insecticidal Nets (LLINs) are the most sustainable and effective malaria control tool currently available. Global targets are for ≥80% of the population living in malaria endemic areas to have access to and use a LLIN. However, current access to LLINs in endemic areas is 56% due to system inefficiencies and budget limitations. Thus, cost-effective approaches to maximize access of effective LLINs in endemic areas are required. This study evaluated whether LLINs that had been stored for more than five years under manufacturer recommended conditions may be optimally effective against *Anopheles* mosquitoes, to inform malaria control programs and governments on the periods over which LLINs may be stored between distributions, in an effort to maximise use of available LLINs. Standard World Health Organization (WHO) bioassays (cone and tunnel tests) were used to evaluate the bio-efficacy and wash resistance of Olyset® and DawaPlus® 2.0 (rebranded Tsara® Soft) LLINs after five years of storage at 25°C - 33.4°C and 40%-100% relative humidity. In addition, a small scale Ifakara Ambient Chamber test (I-ACT) was conducted to compare bio-efficacy of one long stored LLIN to one new LLIN of the same brand, unwashed and washed. Long-lasting insecticidal nets were evaluated using laboratory reared fully susceptible *Anopheles gambiae sensu stricto* (*s.s*) *(*Ifakara strain) and pyrethroid resistant *Anopheles arabiensis* (Kingani strain). After five years of storage, both unwashed and washed Olyset® and DawaPlus® 2.0 LLINs passed WHO efficacy criteria on Knockdown (KD60) ≥95%, 24-hr mortality ≥ 80% and ≥90% blood-feeding inhibition as per conducted WHO bioassays against susceptible *An. gambiae s.s*. The DawaPlus® 2.0 LLINs also passed combined WHO bio-assay criteria against resistant *An. arabiensis*. Confirmatory I-ACT test using whole nets demonstrated that long stored LLINs showed similar efficacy to new LLINs on both feeding inhibition and mortality endpoints against susceptible and resistant strains. Therefore, even after long storage of around 5 years, both Olyset® and DawaPlus® 2.0 remain efficacious against susceptible *Anopheles* mosquitoes at optimal storage range of 25°C - 33.4°C and 40%-100% relative humidity measured by standard WHO methods. DawaPlus® 2.0 remained efficacious against pyrethroid-resistant strain.

Keywords: Long storage nets, long lasting insecticidal nets, LLIN, ITN, Malaria, Tanzania

DECLARATION

I, **Jeremiah John Musa,** hereby do declare that, the dissertation submitted at the Institution for the requirements of the fulfilment of Master Degree of Science in Public health Research is my own. The scholar's work whenever referred has been acknowledged. I, do declare that; the dissertation is not submitted anywhere else other than Nelson Mandela - African Institution of Science and Technology for academic purposes.

_____ _____

Jeremiah John Musa **Date**

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CERTIFICATION

The undersigned certify that they have read and hereby confirm that the dissertation entitled **"Bio-efficacy evaluation of Long-Lasting Insecticidal Nets after five years of storage: Implications for malaria control programmes"** submitted by Jeremiah John Musa to Nelson Mandela African Institution of Science and Technology, Tanzania in fulfillment of the requirements for the award of Master of Science degree in Public Health Research is a trustworthy work done under our supervision.

_____ _____

Prof. Sarah J Moore **Date**

_____ _____

Dr. Revocatus L. Machunda **Date**

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DEDICATION

I dedicate this work to my family, parents, mentors and supervisors: Professor Sarah Moore (IHI), Dr. Revocatus L. Machunda (NM-AIST), Professor Fredros Okech Okumu (IHI), Professor Nicodemus Govella (IHI), Dr. Kafuruki Shubis (IHI), Dr. Issa Lyimo (IHI), Dr. Dickson Lwetojela (IHI), Dr. David O. Nyakundi (MWECAU), Dr. Japhet Chilongola (KCMC). Also, I dedicate this work to IHI-VCPTU, IHI- Training unity and entire IHI community and Nelson Mandela African Institution of Science and Technology (NM-AIST).

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ACT	Artemisinin-based Combination therapy
ANC	Antenatal clinic
EPI	Expanded Programme of immunization
CI	Confidence Interval
CIPAC	Collaborative International Pesticide Analytical Council
GMP	Global Malaria Programme
I-ACT	Ifakara Ambient Chamber Test
IHI	Ifakara Health Institute
IRB	Institutional Review Board
IRS	Indoor Residual Spraying
KD60	Mosquito Knockdown at 60 minutes
LLINs	Long-Lasting Insecticidal Nets
LS	Longs storage
NGOs	Non-Governmental Organizations
NIMR	National Institute for Medical Research
NM-AIST	Nelson Mandela African Institution of Science and Technology
NMCP	National Malaria Control Program
OR	Odds ratio
PBO	Piperonyl -butoxide
PMI	President's Malaria Initiative
TNVS	Tanzania National Voucher Scheme

UNICEF	United Nations Children's Fund
USAID	United State Agency for International Development
UV	Ultra violet rays
VCPTU	Vector Control Product Testing Unit
WHO	World Health Organization
WHO-PQ	World Health Organization Prequalification

CHAPTER ONE

INTRODUCTION

1.1Background of the Problem

Malaria remains a public health problem globally. Deaths attributed by malaria were 435 000 and 219 million cases globally in 2018 (WHO, 2018). Despite several interventions employed the global trend on malaria cases is reported to be increasing in recent years (WHO, 2018). The largest burden of 93% malaria deaths and 92% malaria cases remain in Africa (WHO, 2018). Among of the various interventions commonly used to fight malaria in endemic areas, are Long-lasting insecticidal nets (LLINs), Indoor-residual spraying (IRS) and drug treatment such Artemisinin-based combination therapy (ACT) (Agossa *et al*., 2018; Pryce, Richardson & Lengeler, 2018), these intervention have played big role on malaria reduction in endemic areas (Bhatt *et al*., 2015).

Despite insecticide resistance, long-lasting insecticidal nets (LLINs) remain the most sustainable and effective malaria control tool available in endemic countries (Kleinschmidt *et al*., 2018; Pryce *et al*., 2018). Approximately 663 million cases of malaria, were prevented by LLINs since the year 2000, representing 68% of the total cases averted by all interventions used for malaria control (Bhatt *et al*., 2015). Mass distribution of LLINs after every three years, was recommended by WHO as the core element of the global malaria strategy for malaria vector control in endemic areas (Khanam *et al*., 2018; Kilian, Koenker & Paintain, 2013; WHO, 2018). Between 2008 to 2016, more than one billion LLINs were distributed in Africa through mass campaigns (Kilian *et al*., 2017). The wide scale up of LLINs distributions has led to significant reduction in malaria morbidity and mortality (Krezanoski, 2016). For a brand of LLIN to be listed as a potential product for mass campaign by the WHO, it must undergo rigorous testing from laboratory (phase I) to field testing (phase II) (WHO, 2013a). Currently, there are twenty brands of LLINs that are prequalified by the WHO for use in national distribution campaigns (WHO-PQ, 2018). These LLINs are expected to retain their insecticidal activity for at least 3 years (20 washes is used as a proxy for 3 years of use), by killing mosquitoes and preventing mosquito bites, to give personal and community protection from vector borne diseases (WHO, 2013a).

The public health benefit of LLINs is attained through sustained high net access at the community level, which is referred to as universal coverage (WHO, 2019). The global target for population access to LLINs is ≥ 80%, and is referred to as the minimum operational effectiveness coverage level that would translate the impact of LLINs in the community, thus cases and deaths due to malaria could be significantly reduced (WHO, 2014a, 2015a). Operationally, this is defined as one net used per two people (defacto) in the population (Koenker *et al.*, 2018; WHO/GMP, 2017).

The governments of endemic countries and international donors, such as Global Fund, President's Malaria Initiative (PMI), as well as non-governmental organisations (NGOs) have been providing funds for procurement of LLINs and related logistics to ensure high access to LLINs through multiple channels (AMP, 2017). Nevertheless, current access to LLINs is 56% in endemic areas (WHO, 2018). Even shortly after mass distribution campaigns of LLINs, population access rarely exceeds 80% (Kilian *et al*., 2017; Koenker, 2018). In Tanzania, access to LLINs is 50% (WHO, 2018). Insufficient access to LLINs is mainly due to long intervals between net distribution campaigns, population growth, inadequate funds and budget limitations on malaria control programs (Gomes de Mattos, Oliveira, Leiras, Baptista de Paula Filho & Gonçalves, 2018; Khanam *et al*., 2018; Koenker, 2018; WHO, 2018). Increasing access to LLINs through cost-effective solutions remains a critical concern and a number of strategies are being explored for "keep up campaigns" to retain high LLIN access (WHO/GMP, 2017).

The logistics involved in mass distribution campaigns are enormous, and were estimated to be 7% of total costs of LLIN procurement and delivery in Tanzania (Bonner *et al*., 2011). It is also known that the correct storage of nets is important in retaining their bio-efficacy, before and during mass distribution campaigns. Exposure of LLINs to direct sunlight (Karakuş *et al.*, 2016) and storage at high temperature will degrade the pyrethroid insecticides used on LLINs (Atieli, Munga, Ofulla & Vulule, 2010; Karakuş *et al*., 2016) and guidance on the correct storage conditions for LLINs before and during distributions is available (USAID, 2014). However, there is limited information on the maximum storage period for LLINs before they are no longer bio-efficacious.

1.2 Statement of the Problem

Despite various efforts and approaches used in malaria endemic areas to maximize access to LLINs, the number of LLINs distributed is not enough due to a number of factors including long interval between LLINs distribution campaigns, population growth, inadequate funds and limited budget. However, the effective use and appropriate storage of available LLINs is important, nevertheless, it is not known how long the LLINs may be stored between distributions. Therefore, this study evaluated the bio-efficacy and wash resistance of Olyset® and DawaPlus® 2.0 LLINs (rebranded Tsara soft), that had been stored for more than five years (long storage; LS) under optimal storage conditions at an average temperature of 29°C [25°C - 33.4°C] and 40% - 100% relative humidity (RH).

1.3 Rationale of the Study

Maximizing the access (own) and use of LLINs (≥80%), is necessary to translate the communal level effect of LLINs through reducing cases and deaths due to malaria. After every three years, National Malaria Control Programmes (NMCPs) of endemic countries, conduct mass distributions campaigns for universal coverage of LLINs to increase access. Prior to distribution, a bulk of nets are stored in facilities, however, it is not known how long the LLINs may be stored before distributions. Maintaining optimal storage conditions (Temperature and Humidity) of LLINs for continuous distribution of LLINs is necessary to retain their bio-efficacy. Therefore, there was a need to understand the bio-efficacy and the period over which the LS nets may be stored between distributions, to maximize coverage for sustained malaria control.

1.4 Objectives

1.4.1 General objective

To evaluate the bio-efficacy and wash resistance of long storage Olyset® and DawaPlus® 2.0 LLINs after five years storage under optimal storage conditions at an average temperature of 29°C [25°C - 33.4°C] and 40% - 100% relative humidity.

1.4.2 Specific objectives

(i) To determine the bio-efficacy and wash resistance of LS Olyset® and DawaPlus® 2.0 LLINs washed 0, 1, 3, 5, 10, 15, and 20 times in the laboratory (Phase I).

(ii) To determine the bio-efficacy and wash resistance of whole net, LS Olyset® and DawaPlus® 2.0 LLINs compared to new unwashed and washed 20 times LLINs of the same brands in Ifakara-Ambient Chamber Test.

1.5 Research questions

(i) What is the bio-efficacy and wash resistance of LS after five years of storage under optimal storage condition?

(ii) Are there differences in bio-efficacy and wash resistance of long storage when compared to new LLINs?

1.6 Hypothesis

(i) **Null hypothesis (H_0):** There is no difference in bio-efficacy and wash resistance between the new and long storage LLINs against susceptible and resistant mosquito strain.

(ii) **Alternative hypothesis (H_1):** There is a difference in bio-efficacy between the new and long storage LLINs against susceptible and resistant mosquito strain.

1.7 Significance of the study

The study provide useful information to the NMCPs, other governmental departments of endemic areas, International funders, NGOs and other stakeholders involved in malaria control programmes on long-term storage, storage facilities and conditions against LLINs bio-efficacy in order to make informed decision when procuring LLINs.

1.8 Delineation of the study

Several studies have reported on the bio-efficacy, fabric integrity and residue chemical content of LLINs under user condition (durability studies). But the current study focused only on evaluating the bio-efficacy of LLINs stored for more than five years under optimal storage conditions, without domestic use, in order to suggest a cost effective approach for maximizing access of LLINs in endemic areas for sustained malaria control.

CHAPTER TWO

LITERATURE REVIEW

2.1 Long- lasting insecticidal nets (LLINs) and their working principles

Long-lasting insecticidal nets (LLINs) are factory-treated and their insecticides are either incorporated into fibres or coated with a binder. They are expected to retain efficacy for three years or 20 washes (WHO, 2013a). They work by providing physical barrier between mosquito and a person sleeping under. The pyrethroid insecticide and its repellency properties kill and repel mosquitoes that come into contact with the LLIN surface. A person sleeping under LLIN is highly protected from mosquito bites and where there is a high access of LLINs and use in the malaria endemic community, they offer community protection (community level effect of LLINs) (Komazawa *et al*., 2012). Community level effect is a protection of every person in a community due to reduction of population density of malaria vectors by either being killed by insecticide or exhausted during host seeking (Killeen *et al*., 2011; Komazawa *et al.,* 2012). Quantifying the community level effect of LLINs, through mathematical modelling attaining LLINs access of >80% to the population at risk of malaria would protect the entire community (Koenker e*t al.*, 2018).

2.2 Long-lasting insecticidal net delivery approaches in the malaria endemic communities

Mass distribution campaigns were recommended by WHO as the main and cost-effective delivery approach that every member of the household has an access to LLIN (Kilian *et al*., 2017; WHO/GMP, 2017). However, several approaches have been also suggested and are used as strategy to maximize access to LLINs depending on individual country programs and feasibility. These include, routine channels, such as antenatal and child immunization clinics, school and community based programs, public sector channel (i.e. voucher scheme), church and mosque, agriculture and food supply scheme and work place (WHO/GMP, 2017; WHO, 2013b). These channels have increased LLIN accessibility and use in endemic areas (Omonijo, 2019), although they are not enough to reach the global targets. Globally, 85% of LLINs were distributed through free mass distribution campaigns, 8% in antenatal (ANC) care facilities and 4% as part of immunization programs (EPI) (WHO, 2018). Kramer *et al*. (2017) in Tanzania and Raghavendra

et al. (2017) in India reported that, the use of Tanzania National Voucher Scheme (TNVS), was highly innovative approach that promoted effective and equitable distributing of LLINs for protection of the population at risk of malaria (Kramer *et al*., 2017; Raghavendra *et al*., 2017). However, the TNVS was abolished due to fraud and corruption in 2014 (RBM, 2016).

2.3 Availability of LLINs in malaria endemic countries

To ensure sustainable availability of LLINs in malaria endemic areas, NMCPs with the support from WHO have been using several initiatives for the benefits of people at risk of malaria. International donors and governments of the endemic areas have been great funders of LLINs (WHO, 2018). United States Agency for International Development (USAID/PMI) (Krezanoski, 2016), Global fund, World Bank and NGOs i.e. Bill and Melinda Gates Foundation are the giant funders for LLINs worldwide (Hoibak, 2019; Krezanoski, 2016; WHO, 2018). According to the WHO report in 2018, 624 million of LLINs were delivered globally in 2017. About 552 million LLINs were distributed by NMCPs, 459 million (83%) of LLINs were delivered in sub-Saharan Africa over the period 2015–2017 (WHO, 2018).

2.4 Resistance of mosquitoes to the effectiveness of LLINs

Pyrethroid is the only class of insecticides recommended by WHO for use on bed nets, due to low human toxicity and effective insecticidal functions for killing and preventing mosquito bites (Ranson *et al.*, 2011). The pyrethroid insecticides have been used for a long period of time for malaria vectors control and agricultural activities (Mahande, Msangi, Lyaruu & Kweka, 2018; Matiya, Philbert, Kidima & Matowo, 2019). Frequent use of pyrethroids have led the mosquitoes to develop resistance mechanism (Matiya *et al*., 2019). Insecticide resistance refers to the ability of an insect to tolerate or adapt the adverse effects and toxicity of an insecticide by means of natural selection or mutations (Ranson *et al*., 2011; Silva, Santos & Martins, 2014) through diverse mechanisms such as metabolic resistance (over-expression of cytochrome P450 genes) and knockdown (Kdr) resistance (Kisinza *et al*., 2017; Ranson *et al*., 2011; Silva e*t al*., 2014).

The insecticide resistance is widely prevalent in Africa (Churcher, Lissenden, Griffin, Worrall & Ranson, 2016; Toto, Adam, Peter & Lines, 2017), including Tanzania (Kabula, 2014; Kisinza *et al*., 2017). Managing pyrethroid resistance and improving the performance of LLINs in areas

with insecticide resistant vectors, new generation of LLINs with active ingredients synergistically (pyrethroid insecticide and Piperonyl-butoxide (PBO)) have been developed. It has been proved that, PBO-LLINs are more effective towards pyrethroid resistant mosquitoes resulting in a 60% reduction in malaria parasite prevalence among users (Protopopoff *et al.*, 2018). Piperonyl-butoxide(PBO) is a biochemical substance that hinders (P450 inhibitor) enzymatic responses of insects against detoxifying pyrethroid for its survival, hence reinstate the killing effect of pyrethroid (Gleave, Lissenden, Richardson & Ranson, 2017; Protopopoff *et al.*, 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Two brands of LLINs: Olyset® and DawaPlus® 2.0 that had been stored for more than five years, denoted as long storage (LS) LLINs under recommended conditions, were evaluated. The study was conducted in two stages. First, through randomized double blinded, bio-efficacy evaluation of LLINs using standard WHO assays (WHO, 2013a). This was followed by a partially randomized double blinded semi field tests to compare the bio-efficacy of LS LLINs to new LLINs of the same brand using the Ifakara Ambient Chamber tests (I-ACT) (Massue *et al*., 2019). Un-treated Safi Net was used as a negative control in all tests to monitor the quality of the experiment.

3.2 Test facility

The experiments were performed at the Vector Control Product Testing Unit (VCPTU) of the Ifakara Health Institute located in Bagamoyo, Tanzania (http://ihi.or.tz/static/media/Vector-Control-Product-Testing.e31c173f.pdf).

3.3 Test nets

Olyset® is a high-density mono-filament polyethylene (HPDE) LLIN, incorporated with 20 g/kg (±3 g/kg), 2% w/w of permethrin (corresponding to 1000 mg/m^2). Olyset® is manufactured by A to Z Textile Mills Ltd, Arusha, Tanzania. DawaPlus® 2.0 LLIN name was changed to Tsara® soft. Tsara soft is a deltamethrin-coated on a knitted multi-filament polyester fiber with target dose of 2.0 g/kg ± 25% with 100-denier yarn (corresponding to 80 mg/m^2). It is manufactured by NRS Moon Netting FZE Pakistan. Safi Net is untreated net made of polyester fibres, manufactured by A to Z Textile Mills Ltd, Arusha, Tanzania. The net was used to control the quality of the experiments. All nets used in this study were double sized and coded by an independent technician, to allow blinding of investigators and participants. All test nets have WHO-PQ listing.

3.4 Net Storage conditions

Long storage Olyset® and DawaPlus® 2.0 LLINs were stored at the Ifakara Health Institute (IHI-Bagamoyo) storage facility. All nets were received directly under similar conditions from the manufacturer and were manufactured shortly before shipping for the purpose of product evaluation. Long storage Olyset® LLINs with batch number L2605 were manufactured in May, 2013 and logged into the IHI-Bagamoyo storage facility on 4th June, 2013. Long storage DawaPlus® 2.0 LLINs were regular production manufactured in November, 2013 and were logged into the IHI-Bagamoyo storage facility on 4th December, 2013.

The new Olyset® LLINs were manufactured in 2017 with batch number 7X15BZS, and were logged into the IHI-Bagamoyo storage facility on 22nd December, 2018. The new DawaPlus® 2.0 LLINs were test series manufactured on March, 2018, with batch number 18SPL005, and were shipped from the manufacturer on 15th May, 2018 and logged into the IHI-Bagamoyo storage facility on 1st June, 2018. All nets were stored and maintained at an average temperature of 29°C [25°C - 33.4°C] and 40% - 100% relative humidity in the IHI-Bagamoyo storage facility. Temperature was recorded and logged each afternoon at 1400 h which coincides with peak temperatures.

The experiments were conducted from 25th January 2019 to July 2019. Olyset® LLINs had been stored for 5 years and 2 months while DawaPlus® 2.0 LLINs had been stored for 4 years and 8 months at the time of WHO cone assays and tunnel testing. Olyset® LLINs had been stored for 5 years and 8 months while DawaPlus® 2.0 LLINs had been stored for 5 years and 2 months at the time of I-ACT testing.

3.5 Net preparation for WHO bio-assays

Eight LLINs (4 nets of each brand) were selected at random from their product batches. LLINs were coded, cut into pieces (25 cm x 25 cm) and washed at 1, 3, 5, 10, 15, 20, and 25 times following WHO standards procedures for phase I (WHO, 2013a). One day washing interval was used based on the reported regeneration time for both products (WHO, 2009).

3.6 Net pieces cutting procedures for WHO cone bio-assay

Four of each Olyset® and DawaPlus® 2.0. Thirty-six pieces of 25 cm by 25 cm were cut from Olyset® (nine pieces from each whole net) and equivalent number of pieces were also cut from DawaPlus® 2.0 (nine from each whole net) wrapped in aluminum foil and held at 2-8^{o}C and stored subsequent to WHO procedures and guidelines (WHO, 2013a). Eight pieces (8) were randomly selected from Olyset® LLINs (two pieces from each net) and equivalent number of net pieces were also randomly selected from DawaPlus® 2.0 LLINs (two from each net) for regeneration time evaluation. The regeneration time of both Olyset® and DawaPlus® 2.0 LLINs was known to be one day (WHO, 2009), therefore, regeneration time evaluation was not conducted in this study. For wash resistance evaluation, 28 pieces were randomly selected from each Olyset® (7 pieces randomly selected from each net) and equivalent number was also randomly selected in DawaPlus® 2.0 LNs (7 pieces from each net).

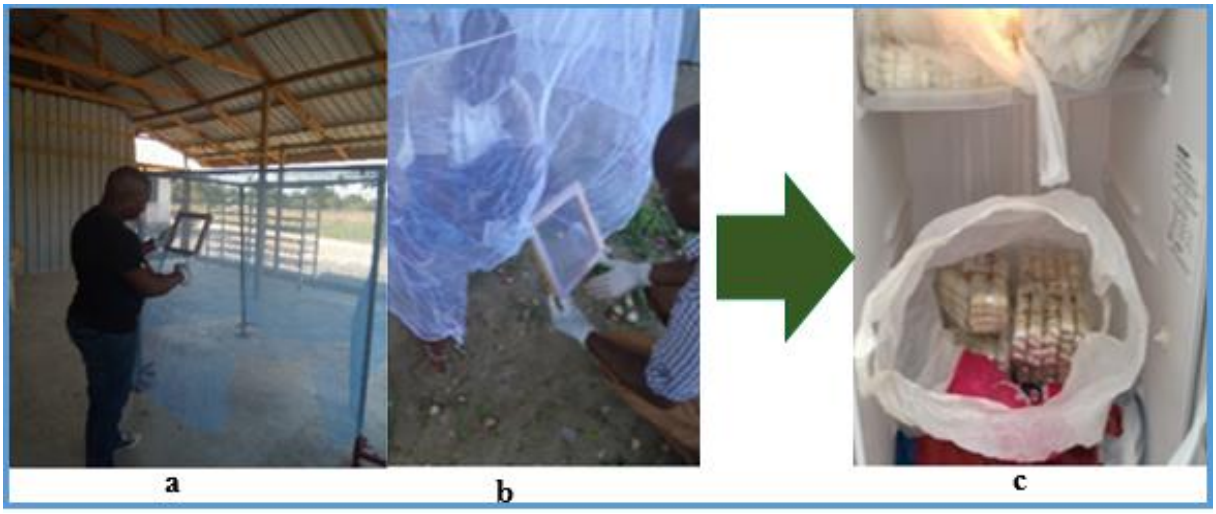

Figure 1: Net piece cutting process and storage

Key:

a = Net pieces cutting process type one

b = Net pieces cutting process type two and

c = Net pieces storage after cutting.

3.6 Washing procedures for WHO bio-assay

Washing removes insecticides from the surface of LLIN, but regenerates after few hours and this is known as regeneration time. Regeneration time is a time taken by LLIN to recover the insecticides after wash. This was done to test the wash resistance of the nets. Net samples; LS Olyset® (28 pieces) and LS DawaPlus® 2.0 (28 pieces) were washed in the laboratory following WHO standards and guideline (WHO, 2013a). Each net sample was individually introduced into 1-litre glass bottles containing 0.5 litre deionized water, with 2.5 ml of the stock Collaborative International Pesticide Analytical Council washing agent (CIPAC), and the bottle was capped with a steel lid and inverted 10 times. The bottle was then placed in a water bath in an upright position for 10 minutes (Fig. 2a), after which the piece of netting was removed with tweezers and excess fluid removed by gently shaking. After washing, the piece of netting was added to a 1 litre glass bottle containing 500 ml of de-ionized water at 30°C ± 5°C. The bottle was capped with a steel lid, inverted 10 times and the placed in a water bath in an upright position for 10 minutes again. After 10 minutes, the net sample was removed using tweezers. This procedure was repeated for third time, and then after the second rinse, the net sample was gently shaken to remove excess water and then allowed to dry on a line for 30 minutes at room temperature (27°C ± 5) out of direct sunlight (Fig. 2b). Once dry, the net samples are wrapped in aluminium foil and stored at 30°C in the incubator until the next wash.

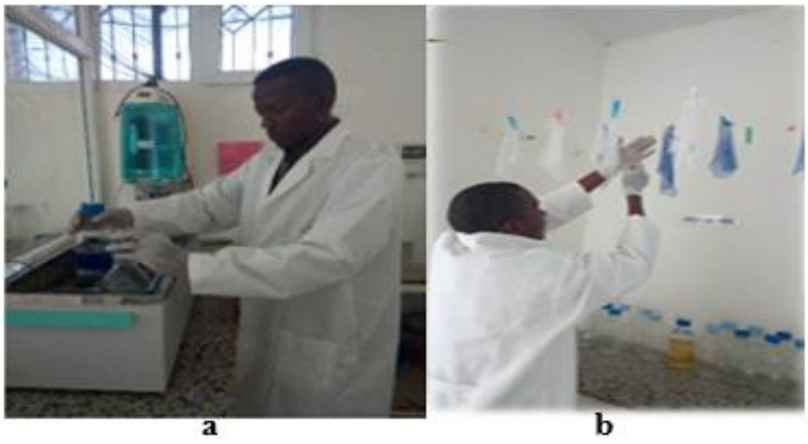

Figure 2: Net pieces washing process in the laboratory

Key:

a = Technician washing individual net pieces in a 1 L bottle and b = Technician lining net pieces after wash.

3.7 Net preparation for I-ACT assays

Eight LLINs (2 old and 2 new DawaPlus® 2.0 and 2 old and 2 new Olyset®) and 2- untreated Safi nets were randomly selected from their product batches and coded. Two LLINs of each brand were washed 20 times as per WHO phase II washing procedures as a standard procedure to simulate aging of nets under user conditions (WHO, 2013a), while the other two were unwashed. All washed, unwashed and un-treated Safi nets were deliberately holed 6 times with 4 cm by 4 cm with one hole on each width and two holes on each length side, 75 cm from the top of the net (half way) as per WHO procedures (WHO, 2013a).

3.8 Net washing procedures for semi-field testing (I-ACT)

Each net was independently washed in an aluminium bowl containing 10 L of IHI bore well water and 20 g dissolved of JAMAA soap (2 g/litre). Each net was washed, by stirring it using heavy duty gloved hand for 3 minutes at 20 rotations per minute (r/min). After that a net was left to soak for 4 minutes. Then the net was removed from the bowl after 4 minutes and hung over a line, and the bowl was emptied. Another 10 L of fresh water was added in the bowl and the net was added and stirred for 2 minutes at 20 r/min, followed then by removal from the bowl and hang over the nylon line for the second time. After emptying the bowl, another 10 L of fresh water was added and the net was put back and stirred for 2 minutes at 20 r/min. Finally, the net was lined for drying in a shade. The net was then repacked in the foil after drying, labeled and stored at 27+/-2 until next wash. Each net was washed once per day, for 20 days consecutively. Then the nets were holed deliberately before the experiments as explained at section 3.7 above, shown in (Fig. 3) below.

a b

Figure 3: Phase two net washing process

Key:

a = Technicians washing whole net and b = Technicians drying of whole net under shade

3.9.1 Test systems

The study used *Anopheles gambiae* (Ifakara strain) fully susceptible to all classes of insecticides and *Anopheles arabiensis* (Kingani strain) resistant to all pyrethroids, including deltamethrin and permethrin (<20% mortality with WHO discriminating doses, metabolic CYP450 mechanism). Three to five days old female sugar fed mosquitos were used in cone bioassays, while 5-8 days old female sugar starved mosquitos were used in the tunnel test and I-ACT. The VCPTU mosquito colonies were maintained at 27°C ± 5 and 40% - 100% relative humidity, with access to 10% sucrose *ad libitum* supplemented by membrane feeding using cow blood for the purposes of egg laying following MR4 guidelines (Kaufmann, 2014).

3.9.2 World Health Organization Bio-assay

(i) Cone bioassay procedures

The standard WHO cone bioassay was used to determine the bio-efficacy and wash resistance of long stored LLINs: Olyset® and DawaPlus® 2.0. On each net sample standard WHO cone was placed and held in place using a plastic manifold. This was conducted to the unwashed and washed for 1, 3, 5, 10, 15, 20, and 25 times at one-day intervals. Five laboratory-bred susceptible *An. gambiae s.s* (Ifakara strain) mosquitoes, sugar fed of 3-5 day old were put into each cone and exposed for 3 minutes. The procedure was repeated to resistant *An. arabiensis* (Kingani strain) strongly resistant to deltamethrin (<20% mortality) following WHO guideline (WHO, 2013a).

After the exposure, mosquitoes were removed gently from the cones and kept separately in paper cups using siphon and provided with cotton wool moistened with 10% sucrose solution. The Outcome measurement were the proportion of mosquitoes Knockdown after 60 minutes (KD60) and mortality after 24-hr (Table 1). Long storage LLINs that failed to meet WHO efficacy criteria (i.e. ≥95% KD60 and ≥80% 24-hr mortality) in the standard WHO cone bioassay were subjected to WHO tunnel test (WHO, 2013a) (Table 1, Fig. 4). Each test replicate and associated control was tested with both the susceptible and resistant strain.

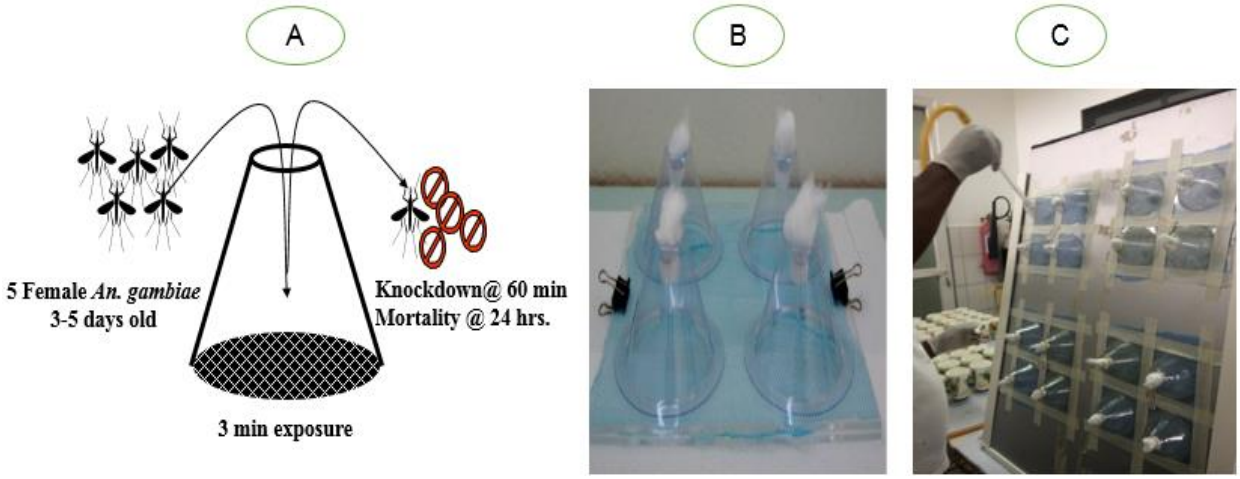

Figure 4: Cone bio-assay procedures

Key:
A = Schematic diagram of WHO cone bio-assays
B = Standard cone placed on net piece
C = Releasing mosquitoes in each cone for 3 minutes exposure on each net type.

(ii) WHO Tunnel test procedures.

WHO Tunnel test was used to assess blood-feeding inhibition through comparing the proportion of blood-fed females (alive or dead) in treatment and control tunnels. Overall mortality was measured by pooling the mortalities of mosquitoes from the two sections of the tunnel. Both unwashed and washed. Only one of the four net pieces, which gave mortality closest to the average mortality in the cone bioassay was selected. At 1800 h, in each WHO tunnel, one hundred mosquitoes were released per tunnel. Each piece was tested with both the susceptible and resistant strains on separate occasions. A control for each of the strain was used (WHO, 2013a) (Table 1). The mode and structure of the WHO tunnel was followed as per WHO guideline and as explained by other authors (Massue *et al*., 2019; WHO, 2013a). A restrained rabbit bait unable to move was

introduced in the cage at the end of the longer section of the tunnel. The next morning at 0900 h mosquitoes were removed using a mouth aspirator and counted separately from each section of the tunnel. Blood feeding inhibition and 24-hr mortality were the outcome measures (Table 1, Fig. 5).

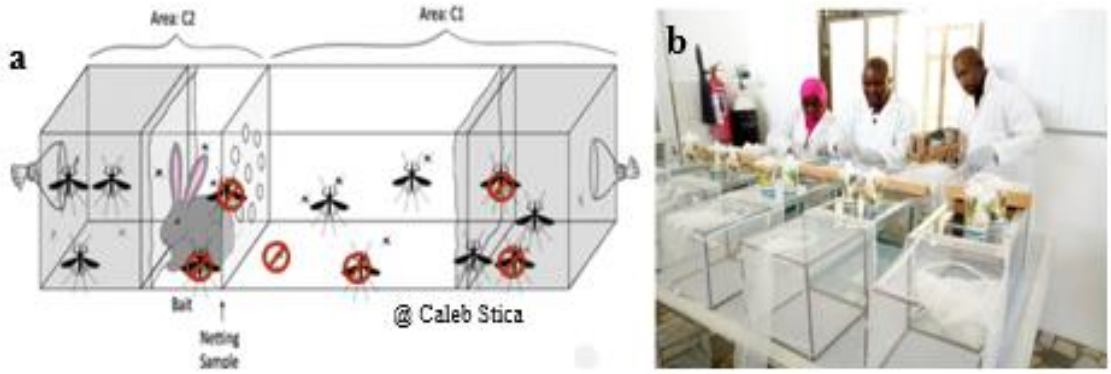

Figure 5: Tunnel test process

Key:

a = Schematic diagram of WHO tunnel with rabbit as a bait and b = Technicians setting up WHO tunnel test experiment.

3.9.3 Ifakara Ambient Chamber Test assay

I-ACT was used as an intermediate between laboratory and experimental hut tests (Massue *et al*., 2019). One LLIN per condition (unwashed or 20 times washed) was tested. Each LLIN and control was randomly assigned to one of the ten testing chambers of the I-ACT (Fig. 1a). At 2100 h, 30 *An. gambiae and* 30 *An. arabiensis,* were released in each testing chamber. Mosquitoes were lightly dusted with fluorescent powder (SWADA, Cheshire, United Kingdom) to distinguish the strain as they are morphologically identical. At 0630 h, mosquitoes were collected into paper cups using a mouth aspirator. Mosquitoes were scored immediately after collection by strain and four categories: a) dead and unfed, b) dead and blood-fed, c) alive and unfed or d) alive and blood-fed. Mosquitoes were then held in the testing laboratory at 27°C ± 5 and 40% - 100% relative humidity with 10% sugar solution. After 24-hr, the proportion of mosquitoes in each of the four categories was again scored. After each experiment, test nets were re-packed in their respective bags, chambers were cleaned and bed sheets were washed. Long-lasting insecticidal nets

remained fixed to their respective chambers while volunteers rotated nightly between chambers, for ten experimental nights so that each volunteer tested each net type once. This was done to account for difference between human attractiveness to mosquitoes that might affect the proportion of mosquito's blood feeding. Acceptable control mortality was ≤ 10% or ≥50% blood-feeding success (WHO, 2013a) (Table 1, Fig. 6).

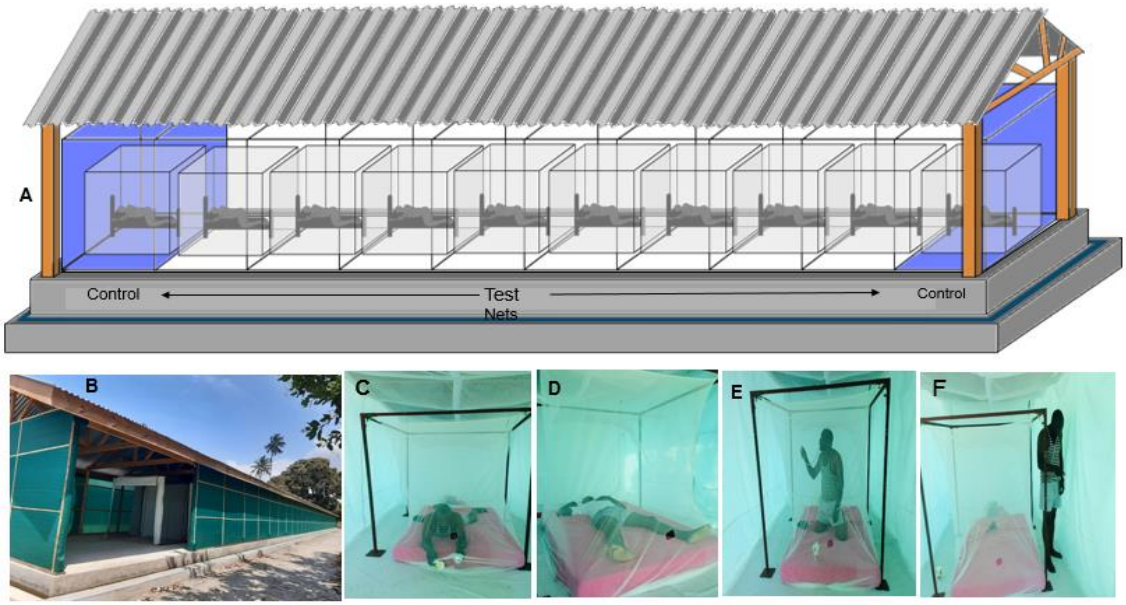

Figure 6:Semi-field system (I-ACT)

Key:

A = Schematic diagram of the Ifakara Ambient Chamber Test (I-ACT with10 chambers)

B = I-ACT at IHI-Bagamoyo branch

C= Volunteer releasing mosquito within chamber outside the net;

D= Volunteer sleeping in side net within a chamber;

E= Volunteer collecting mosquitoes using mouth aspirator (siphon) inside net within chamber.

F= Volunteer collecting mosquitoes using siphon outside net within chamber.

Table 1: Summary of experimental design on WHO and I-ACT Bioassays

Particular	WHO Cone test	WHO tunnel test	Ifakara Ambient chamber test (I-ACT)
Mosquitoes exposed	80 per net	100 per net piece	60 (30 per strain) per net
Exposure time	3 minutes	12 hours	9 hours
Mosquito holding conditions	27°C ± 5°C 40% - 100% RH	27°C ± 5°C 40% - 100% RH	27°C ± 5°C 40% - 100% RH
Mosquito status	3-5 days female Nulliparous, sugar fed	5-8 days female, sugar starved, nulliparous	5-8 days female, sugar starved, nulliparous
Bait	None	Rabbit	Human
Outcome measures	% KD60 % 24-hr mortality	% Feeding inhibition % 24-hr mortality	% Feeding inhibition % 24-hr mortality
WHO efficacy criteria	≥95% KD60 ≥80% 24-hr mortality	≥90% Feeding inhibition ≥80% 24-hr mortality	≥90% Feeding inhibition ≥80% 24-hr mortality
Test validity Control	≤10% mortality	≥50% feeding success ≤10% mortality	≥50% feeding success ≤10% mortality
Analysis	Descriptive analysis	Descriptive analysis	Descriptive analysis & Binary logistic regression

3.9.4 Data management and analysis

Data were recorded on paper forms, double entered into Microsoft excel 2013 and cleaned prior to analysis. Data analysis was performed using STATA 13.1. Descriptive statistics were used for WHO cone and tunnel tests. For I-ACT, both descriptive statistics and mixed effect binary logistic regression were conducted. The outcome measures were 24-hr mortality and blood feeding inhibition. Model fit was tested using AIC (Shi & Tsai, 2002). For the model with mortality as the outcome, the best fitting model had treatment and volunteer as fixed effect and day as a random effect while best model with feeding success as the outcome had treatment as a fixed effect, with both volunteer and day as random effects.

3.9.5 Ethical approval and volunteer's protection

Ethical approval was granted by National Institute of Medical research (NIMR/HQ/R. 8a/VIX /115 and Institutional Review Board of the Ifakara Health Institute (IHI/IRB/No: 19-2013 and IHI/IRB/No: 04 - 2019). Human volunteers for net washing and I-ACT were recruited upon a written informed consent that explained the purpose and procedures of the study as well as their roles. Compensation was provided to sleeping volunteers for their time away from home and all the participants were trained on study standard operating procedures (SOPs).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.2 World Health Organization assays results against susceptible *An. gambiae s.s*

Long storage Olyset® LLIN stored for 5 years and 2 months (long storage, LS) fulfilled WHO bio-efficacy criteria up to 20 washes based on the combined WHO Cone bioassay and tunnel test against susceptible *An. gambiae* s.s. (Table 2). Long storage Olyset® LLIN, demonstrated 95% KD60 up to 10 washes in cone bioassay (Fig. 7a) and >90% feeding inhibition up to 20 washes in tunnel tests (Fig. 2d). Mortality was low in cone bioassays (Fig. 7b).

Long storage DawaPlus® 2.0 LLIN stored for 4 years and 8 months fulfilled WHO bio-efficacy criteria up to 20 washes based on cone bioassay against susceptible *An. gambiae* s.s. (Table 2). Long storage DawaPlus® 2.0 LLIN, demonstrated 100% KD60 up to 20 washes (Fig. 7a) and >90% 24-hr mortality up to 20 washes (Fig. 7b).

4.2 World Health Organization assays results against resistant *An. arabiensis*

Long storage Olyset® LLIN did not fulfil WHO efficacy criteria up to 20 washes based on the combined WHO Cone bioassay and tunnel test against resistant *An. arabiensis* (Table 2). Long storage Olyset® LLIN did not approach the 95% KD60 threshold in cone tests, as well as >80% 24-hr mortality (Fig. 8a and Fig. 8c). In the tunnel tests, LS Olyset® LLIN did not approach the 90% feeding inhibition in all tests, except nets washed 3 and 15 times demonstrated >90% feeding inhibition (Fig. 8d). Long storage Olyset® did not generate 80% 24-hr mortality up to 20 washes in both cone and tunnel tests (Fig. 8c).

Long storage DawaPlus® 2.0 LLIN fulfilled WHO bio-efficacy criteria up to 20 washes based on the combined WHO Cone bioassay and tunnel test against resistant *An. arabiensis* (Table 2). Long storage DawaPlus® 2.0 LLIN, either demonstrated >95% KD60 (Fig. 7a) in cone bioassay or >90% feeding inhibition (Fig. 8d). It did not demonstrate 80% 24-hr mortality up to 20 washes in both cone and tunnel tests (Fig. 8c).

Table 2: World Health Organization bio-assays results against Anopheles mosquito strains

Test system	Test item	Washes	Cone test (N= 80)		WHO Tunnel test (N= 100)		Pass/Fail WHO efficacy criteria (2013)
			% KD60 [95% CI]	% 24-hr Mortality [95% CI]	% Feeding Inhibition [95% CI]	% 24-hr Mortality [95% CI]	
Susceptible *Anopheles gambiae* s.s (Ifakara strain)	Olyset®	0	100	03.75 [01.47-06.03]	-	-	Pass
		1	100	31.64 [29.40-33.89]	-	-	Pass
		3	96.25 [95.06 – 97.44]	01.25 [00.06-02.44]	-	-	Pass
		5	93.75 [89.24 – 98.26]	0	-	-	Pass
		10	93.75 [92.56 – 94.94]	02.50 [01.12-03.88]	-	-	Pass
		15	83.75 [79.68 – 87.82]	01.25 [00.06-02.44]	100	90.91	Pass
		20	75.00 [69.85 – 80.15]	03.75 [01.47-06.03]	96.00	51.52	Pass
	DawaPlus® 2.0	0	100	92.50 [90.12-94.88]	-	-	Pass
		1	100	100	-	-	Pass
		3	100	98.75 [97.56- 99.94]	-	-	Pass
		5	100	97.50 [96.12-98.88]	-	-	Pass
		10	100	96.25 [92.68-99.80]	-	-	Pass
		15	100	93.75 [87.79-99.71]	-	-	Pass
		20	100	100	-	-	Pass
Resistant *Anopheles arabiensis* (Kingani)	Olyset®	0	38.75 [34.68 - 42.82]	0	-	-	-
		1	25.00 [19.85 - 30.15]	01.25 [00.06 - 2.44]	-	-	-
		3	23.75 [19.68 - 27.82]	03.75 [01.47 - 6.03]	96.00	09.18	Pass
		5	51.25 [47.18 - 55.32]	03.75 [02.56 - 4.94]	87.00	12.24	Fail
		10	36.25 [33.25 - 39.25]	01.25 [00.06 - 2.44]	87.00	14.29	Fail
		15	53.75 [40.79 - 66.71]	0	92.00	19.39	Pass
		20	58.75 [57.56 - 59.94]	0	88.00	22.45	Fail
	DawaPlus® 2.0	0	50.00 [44.85 - 55.15]	10.00 [05.64-14.35]	-	-	-
		1	63.75 [61.47 - 66.03]	11.25 [06.74-15.76]	-	-	-
		3	67.50 [63.37 - 71.63]	16.25 [15.06-17.44]	90.00	61.62	Pass
		5	91.25 [87.68 - 94.82]	36.25 [31.34-41.16]	94.00	56.57	Pass
		10	100	23.75 [19.68-27.82]	97.00	78.79	Pass
		15	97.50 [96.12 - 98.88]	05.00 [01.63-08.37]	68.00	51.52	Pass
		20	93.75 [89.24 - 98.26]	12.50 [09.42-15.58]	93.00	41.41	Pass

N= number of mosquitoes released on each test

4.3 Ifakara Ambient Chamber Test results against Susceptible *An. gambiae s.s*

Unwashed and 20 times washed LS Olyset® and DawaPlus® 2.0 against susceptible *An. gambiae s.s*, exceeded the WHO bio-efficacy criteria for tunnel test on 24-hr mortality (≥ 80%) and feeding inhibition (≥90%). Unwashed and 20 times washed LS Olyset® and LS DawaPlus® 2.0 nets performed similar to new nets of the same brand and washing status, showing almost identical measurements of mortality and feeding inhibition (Table 3). Washing the nets 20 times only marginally reduced their efficacy but still induced high mortality and feeding inhibition, with the old nets nearly as efficacious as the new nets. On the mortality endpoint, LS unwashed Olyset® marginally outperformed the new unwashed Olyset®: 99.30% [98.25 - 100] vs 96.28% [93.64 – 98.93], Odds ratio 0.17 [0.04 – 0.79] p=0.024. On the feeding inhibition endpoint, LS DawaPlus® 2.0 20x washed marginally outperformed the new DawaPlus® 2.0 20 times washed: 95.62% [92.81 – 98.42] vs 83.81% [78.98 – 88.64] OR 4.37 [2.67 – 7.15], p<0.0001.

4.4 Ifakara Ambient Chamber Test results against Resistant *An. arabiensis*

Unwashed LS Olyset® and unwashed LS DawaPlus® 2.0 against resistant *An. arabiensis,* exceeded the WHO bio-efficacy criteria for tunnel tests on feeding inhibition (≥90%). All the net types and condition failed to meet WHO bioefficacy criteria on 24-hr mortality (≥ 80%) against the resistant strain. Unwashed and 20x washed Olyset® and DawaPlus® 2.0 LS nets performed in a similar way to new nets of the same brand and washing status on both endpoints showing almost identical mortality and feeding inhibition (Table 3). As was observed with the susceptible strain, on the mortality endpoint, LS unwashed Olyset® marginally outperformed the new unwashed Olyset® 63.40% [47.83 - 78.97] vs 50.31% [33.42 - 67.19], Odds ratio 0.49 [95% CI: 0.33 – 0.72], p<0.0001. On the feeding inhibition endpoint, LS unwashed DawaPlus® 2.0 marginally outperformed the new unwashed Tsara® Soft: 91.57% [88.72 - 94.41] vs 81.78% [75.49 - 88.07], OR 2.55 [1.61 – 4.06], p<0.0001. Additionally, on the feeding inhibition endpoint, LS 20 times washed DawaPlus® 2.0 outperformed the new 20 times washed DawaPlus® 2.0: 83.28% [76.48 - 90.08] vs 59.87% [49.89 - 69.85], OR 4.07 [2.60 – 6.36], p<0.0001.

Table 3: Ifakara Ambient Chamber Test results against mosquito strains

Test system	Test item	%24-HRS Mortality* [95% CI]	Odds of dying [95 % CI]	p- value	% Feeding Inhibition§ [95% CI]	Odds of Feeding [95 % CI]	P-value
Susceptible *Anopheles gambiae* s.s. (Ifakara strain)	LS[a] Olyset® unwashed	99.30 [98.25 – 100.0]	1.00		94.00 [92.76 - 99.11]	1.00	
	New Olyset® unwashed	96.28 [93.64 – 98.93]	0.17 [0.04 – 0.79]	0.024	97.27 [94.84 – 99.69]	0.54 [0.05 – 5.80]	0.610
	LS Olyset® washed	85.73 [76.58 – 94.86]	1.00		91.40 [88.66 – 94.14]	1.00	
	New Olyset® washed	85.44 [74.29 – 96.59]	1.09 [0.61 – 1.93]	0.775	92.29 [88.73 – 95.86]	0.84 [0.35 – 1.99]	0.693
	Old DawaPlus® 2.0 unwashed	99.66 [98.88 – 100.0]	-		96.12 [94.40 – 97.83]	1.00	
	New DawaPlus® 2.0 unwashed	99.65 [98.88 – 100.0]	-	-	89.37 [82.90 – 95.84]	2.14 [0.62 – 7.47]	0.231
	Old DawaPlus® 2.0 washed	100	-		95.62 [92.81 – 98.42]	1.00	
	New DawaPlus® 2.0 washed	96.94 [95.56 – 98.32]	-	-	83.81 [78.98 – 88.64]	4.37 [2.67 – 7.15]	0.0001
Resistant *Anopheles arabiensis* (Kingani strain)	Old Olyset® unwashed	63.40 [47.83 - 78.97]	1.00		92.10 [88.24 - 95.95]	1.00	
	New Olyset® unwashed	50.31 [33.42 - 67.19]	0.49 [0.33 – 0.72]	0.0001	95.16 [91.07 - 99.26]	0.37 [0.08 – 1.76]	0.213
	Old Olyset® washed	33.34 [17.91 - 48.77]	1.00		84.25 [79.51 - 88.99]	1.00	
	New Olyset® washed	37.85 [20.11 - 55.59]	1.18 [0.81 - 1.72]	0.401	86.88 [80.34 - 93.43]	0.67 [0.29 – 1.51]	0.329
	Old DawaPlus® 2.0 unwashed	71.30 [56.28 - 86.32]	1.00		91.57 [88.72 - 94.41]	1.00	
	New DawaPlus® 2.0 unwashed	68.91 [50.64 - 87.19]	0.82 [0.53 – 1.3]	0.364	81.78 [75.49 - 88.07]	2.55 [1.61 – 4.06]	0.0001
	Old DawaPlus® 2.0 washed	48.73 [34.18 - 63.28]	1.00		83.28 [76.48 - 90.08]	1.00	
	New DawaPlus® 2.0 washed	45.74 [27.90 - 63.57]	0.86 [0.60 – 1.22]	0.393	59.87 [49.89 - 69.85]	4.07 [2.60 – 6.36]	0.0001

N= 30 mosquitoes released per strain per test; *Arithmetic mean control corrected mortality with 95% confidence interval (CI) § Arithmetic mean feeding inhibition with 95% confidence interval (CI); [a]LS= long storage

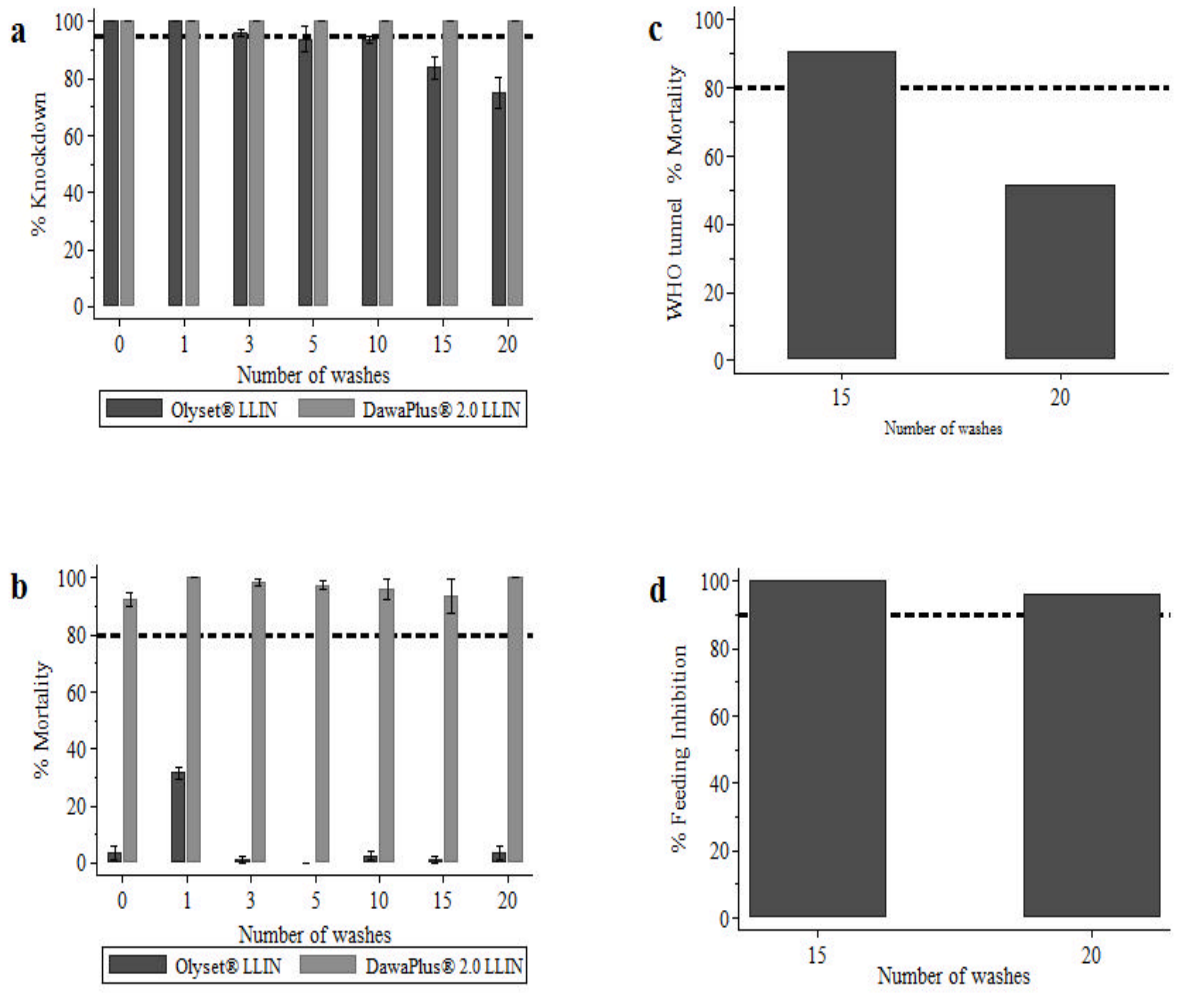

Figure 7: World Health Organization bio-assay results against susceptible *An. gambiae s.s*

Key:

(a) % KD60, **(b)** WHO cone assay % 24-hr mortality, **(c)** Tunnel test % 24-hr mortality and

(d) % Feeding inhibition.

In all graphs (a, b, c and d) the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition

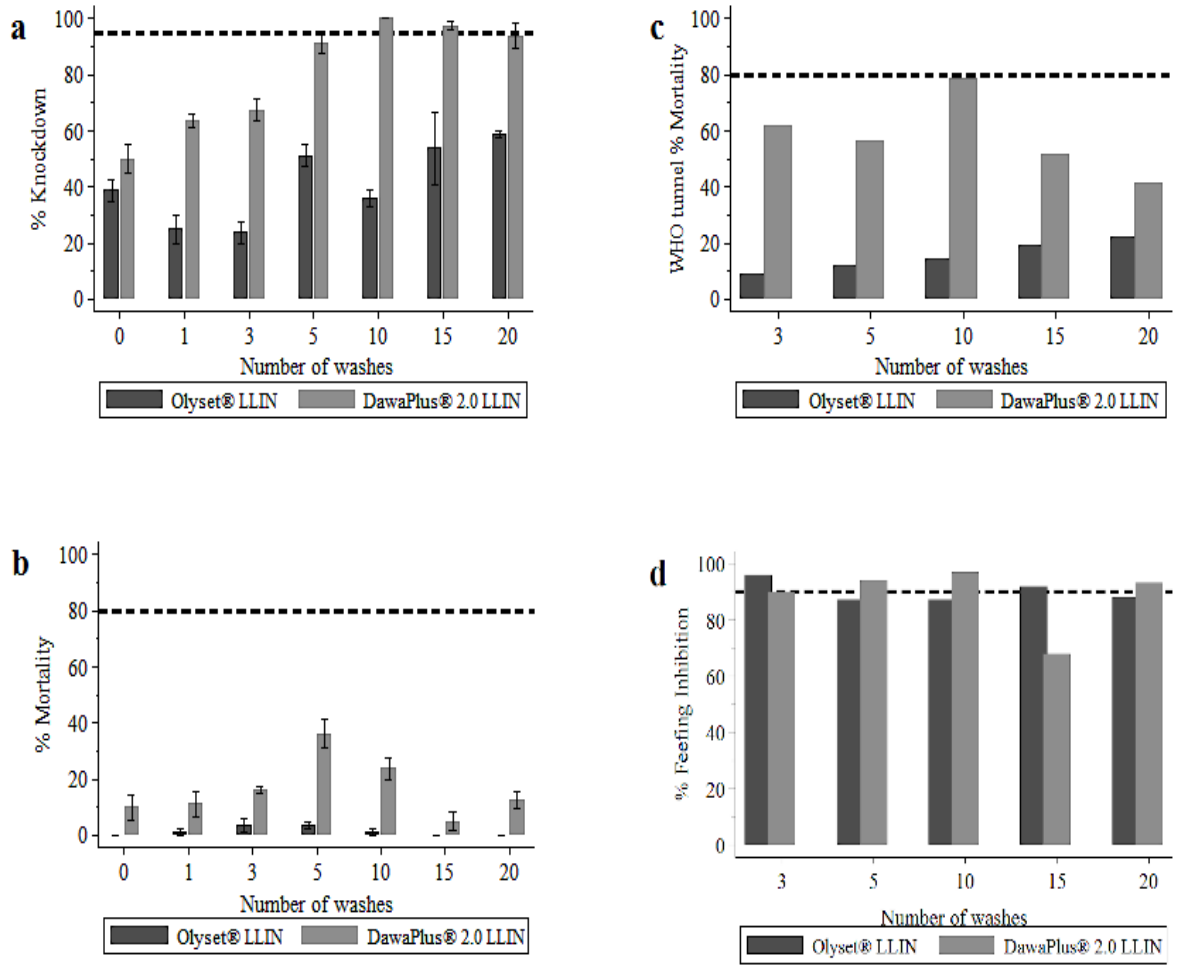

Figure 8: World Health Organization bio-assay results against resistant An. arabiensis

Key:

(a) % KD60, **(b)** WHO cone assay % 24-h mortality, **(c)** Tunnel test % 24-hr mortality and

(**d)** % Feeding inhibition.

In all graphs (a, b, c and d) the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition

4.5 Discussion

(i) Storage conditions of LLINs and Implications to Malaria control programmes

This study provides valuable information on the effect of long storage conditions on the bio-efficacy of LLINs for malaria control programs. The study showed that LLINs remained efficacious despite being stored for more than five years under controlled storage conditions. The nets used for this study were pyrethroid of two types: Olyset®, a permethrin incorporated net, and DawaPlus® 2.0, a deltamethrin coated net with insecticide held to the filaments using a binder.

It was necessary to keep the investigational LLINs under ideal temperature and humidity conditions, as known, high temperature may inactivate the insecticide or binder (Karakuş *et al*., 2016; Peck *et al*., 2014). Proper storage should also avoid direct sunlight as pyrethroids are decomposed by UV light and heat (WHO, 2015b). Several studies have been conducted to evaluate the storage conditions for LLINs for instance, in Turkey by Karakus *et al*. (2016) reported that nets exposed to direct sunlight for six months had lower efficacy (44.4% 24-hr mortality), than other groups of nets which were not exposed to sunlight (100% 24-hr mortality) (Karakuş *et al*., 2016). Atieli *et al.* (2010) showed that drying methods used after washing nets, resulted in significant impact on the efficacy of pyrethroid nets: nets washed 20 times and dried under the shade retained more pyrethroid insecticide (62.5%) than nets directly dried under the sunlight (58.8%) (Atieli *et al*., 2010). Furthermore, Peck *et al.* (2014) reported that the insecticidal activity of the pyrethroid Lambda-cyhalothrin was reduced after 10 weeks of exposure to direct sunlight (Peck *et al*., 2014).

LLINs are designed to withstand high temperatures that may be encountered in the tropics and the findings from this study suggest that nets can retain bio-efficacy for up to five years if stored out of sunlight at the range of 25°C to 33.4°C and 40% - 100% relative humidity. The storage conditions used in this study aligned with the manufacturer specification and WHO guidelines (WHO, 2014b, 2015b). It should be noted that the LLIN store used for this study was a shipping container (Fig. 9) that uses only passive cooling for the majority of the year. The container is raised above the ground and is situated under a second shade roof to reduce the radiant transfer of heat. It is also equipped with ventilation gaps (similar to the eaves of African houses) to allow

air movement through the store. Electric ceiling fans are used only at the hottest times of the year irrespective of the temperature. Therefore, investment in similar storage facility of the Ifakara Health Institute for LLINs can ensure longevity efficacy of LLINs at a low running cost. Also National malaria programmes and other stakeholders should be well informed on the appropriate long-term storage conditions for pyrethroid nets in order for the LLINs to retain their bio-efficacy, if nets are to be stored for extended period before distribution.

Figure 9: The Bagamoyo IHI LLIN storage facility

(ii) Performance of LLINs against Test system and Washes in the WHO bio-assays

The performance of long storage (LS) LLINs varied between net brands and washes in the WHO cone bioassay. DawaPlus® 2.0 LLIN, met the WHO criteria in the standard WHO cone assay without the need to conduct a WHO tunnel test, while Olyset® LLIN failed to meet the criteria based on the cone assay but passed based on WHO tunnel test (Table 2) due to (a) Olyset® is a high density polyethylene, permethrin moves slowly with short wash intervals, hence very low surface concentrations, sufficient to induce KD60 effect, but insufficient to mortality (b) the irritant action of the permethrin insecticide incorporated in Olyset® (Massue *et al*., 2019; Rafinejad *et al*., 2008). This mode of action reduces the probability of mosquito dying from exposure to the insecticide following multiple contacts with net, but also gives Olyset® its feeding inhibition properties that were observed in the I-ACT, allowing protection of human volunteers sleeping beneath them even after 5 years and 2 months of storage. Similar results were observed by Massue *et al.* (2019). It was again observed, by Jaramillo *et al.* (2011), on

which permethrin treated net (Olyset® LLIN) reduced contacts of *An. albimanus* to net surface in the cone test (Jaramillo, Robledo, Mina, Muñoz & Ocampo, 2011).

Both LS Olyset® and DawaPlus® 2.0 failed to meet the WHO mortality efficacy criteria (≥ 80%) against the resistant *An. arabiensis* (Kingani strain), but the nets still performed well on the feeding inhibition end points. As the candidate LLINs utilize pyrethroid insecticides, it is expected the nets to show reduced efficacy against pyrethroid resistant populations, however, it is clear that LLIN performance was not significantly impaired as a result of long storage, but due to ability of the resistant strain to detoxify pyrethroids (Alemayehu *et al*., 2017; Kisinza *et al*., 2017; WHO/ GMP, 2016). It is for this reason that piperonyl butoxide-treated insecticidal nets (PBO) nets have been developed (Protopopoff *et al*., 2018). The PBO is a synergist biochemical substance, combined with pyrethroid, that hinder enzymatic responses of insects against detoxifying pyrethroid for its survival, and allow the pyrethroids insecticide to finally kills pyrethroid resistant mosquitoes (Gleave, Lissenden, Richardson, Choi & Ranson, 2018). Although, it is interesting that both nets still performed well on the feeding inhibition end point, which means that LS-LLINs can still confer protection, therefore reiterate the usefulness in the continuous control of mosquitoes.

(iii) Performance of LLINs against Test system and Washes in the I-ACT

Results from the I-ACT with volunteers sleeping beneath the LLINs complemented the evidence provided by the WHO cone assays and allowed for comparison between new nets and long storage nets of the same brand and washing status. Using WHO pass/fail thresholds, findings from WHO cone assays and the I-ACT with LS nets agreed between net brands and washes. Although, using the WHO criteria, both LS nets and new nets passed with the susceptible strain but inconsistent with the resistant strain in the IACT. Ifakara Ambient Chamber Test (I-ACT), demonstrated higher feeding inhibition and mortality (Table 2, Table 3), than the results obtained in WHO bio-assays. The increased performance of LLINs in the I-ACT might be due to (a) extended exposure time that increased number of contacts between mosquitoes and the LLIN, (b) use of a whole net, (c) use of a preferred (human) bait by mosquito and (d) larger surface are of net presented to the mosquitoes. Similar I-ACT results have also been observed by Massue *et al.* (2019). However, it should be understood from our findings

that, long storage nets performed similarly to the new nets in the I-ACT on both mortality and feeding inhibition.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5. 1 Conclusion

Even after long-term storage of around 5 years, Olyset® and DawaPlus® 2.0 LLINs remained efficacious against susceptible *Anopheles* mosquitoes at optimal storage range of 25°C - 33.4°C for temperature and 40% - 100% relative humidity measured by standard WHO methods. Also, DawaPlus® 2.0 currently known Tsara® soft passed WHO efficacy criteria on unwashed LLINs and after 20 washes against resistant *An. arabiensis.* These data were confirmed in the I-ACT. Therefore, long stored nets can still be useful in controlling malaria in endemic areas.

5.2 Recommendations

This study was conducted as per protocol subsequent to WHO guideline on LLINs evaluation. Therefore, based on the findings obtained in this study, I recommend the following.

(i) Long storage of LLINs at optimal storage conditions at the range of 25°C - 33.4°C for temperature and 40% - 100% RH, does not affect the bio-efficacy of LLINs against *Anopheles* mosquitoes for more than five years.

(ii) Storage condition for LLINs are very important for Programmes in malaria control.

(iii) Provided nets are stored well, they can be stored for use in continuous distribution campaigns to maximize LLINs coverage for sustained malaria control in endemic areas.

(iv) There is a need to evaluate inter-net heterogeneity, proxy for aging and damage (fabric integrity) as well as the bursting strength of long storage nets. It is important as heat may damage net fibres.

(v) There is a need to evaluate the long storage nets in different storage conditions apart from Ifakara Health Institute storage facility to understand variation occurring in net bio-efficacy.

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APPENDICES

Appendix 1. Informed consent for I-ACT volunteers

Information sheet and informed consent for participants sleeping in Ambient Chambers in English

INFORMED CONSENT FORM FOR PARTICIPANTS

Name of Principle Investigator: Mr. Jeremiah John Musa

Name of Organization: Ifakara Health Institute, Bagamoyo, Tanzania

Project code: BIT LS Nets

PART 1. INFORMATION SHEET FOR PARTICIPANTS

Phase II evaluation of Long storage nets compared to new nets of the same brand; DawaPlus® 2.0 LN and Olyset® LN® against strongly pyrethroid resistant *Anopheles arabiensis* and fully pyrethroid susceptible *Anopheles gambiae s.s* (Ifakara strain) in the Ifakara Ambient Chamber test, Tanzania

Introduction

My name is ……. (Name of investigator), I am working for Ifakara Health Institute, Tanzania. We are trying to evaluate the efficacy of long storage nets; DawaPlus® 2.0 LLIN and Olyset® LLIN and compare their efficacy with new nets of the same brand respectively. They are all approved for use by the World Health Organization for malaria control. The Government of Tanzania through the Tropical Pesticides Research Institute (TPRI) has also approved the nets for us to research in Tanzania.

Purpose of research

Malaria is one of the most important diseases in Tanzania. It is spread from one person to another through infected bites of certain mosquitoes. These mosquitoes normally bite at night. It has been shown that sleeping under mosquito nets can help to avoid getting bitten in the night. Furthermore, if the nets are treated with some chemicals that kill insects (insecticides), then they will prevent the bites and also kill the mosquitoes. Therefore, if everybody in the community sleeps under insecticide treated nets, they provide control of the mosquitoes. Some kinds of nets are given a special chemical treatment in the factory and do not require re-

treatment until the end of their useful life; these are called long-lasting insecticidal mosquito nets (LLINs). DawaPlus® 2.0 (rebranded Tsara Soft) and Olyset® are names of such long-lasting insecticidal mosquito net brands.

There is a need to evaluate the efficacy of unwashed and washed long storage nets: DawaPlus® 2.0 and Olyset® in the Ambient Chambers, to find out if they remain effective against malaria-transmitting mosquitoes after they have been stored for a long period of time without domestic use (> 5 years) and compared their efficacy with new unwashed and washed nets of the same brand respectively.

We would like to invite you as a participant in this study. Two types of nets will be tested in the Ambient Chambers to find out their mosquito killing/malaria prevention properties. As a participant you will sleep under the bed nets in the Ambient Chambers. These have holes in them. Then you will be required to collect mosquitoes from your chamber each morning. Neither our research team nor you know which net which is, but it will either be DawaPlus® 2.0 or Olyset®.

Information on study nets

In this study selected nets are factory-treated ones. DawaPlus® 2.0 (currently Tsara soft) is manufactured by NRS Moon Netting FZE, Pakistan and Olyset® is manufactured by A to Z Textile Mills Limited, Arusha Tanzania. These products have been tested by the World Health Organization (WHO) and Tanzania Pesticide Research Institute (TPRI) and are recommended as being safe and effective against malaria for all people to use.

Type of Research Intervention and procedures

- As a participant you will be asked to sleep under the bed nets between 2100 h and 0630 h.
- The work will be done in the screen house tunnel with laboratory reared mosquitoes free of malaria parasites, even if they bite you, you will not get a disease from them.
- You will be asked to sleep in a compartment in the semi-field tunnel. Mosquitoes will be released into the compartment.
- The bed nets are safe for use on humans and have been approved by the Tropical Pesticide Research Institute.
- You will be asked to collect the mosquitoes in the compartment at 0630 h each morning using a siphon.
- You will be asked to not smoke cigarettes or drink alcohol for the days or weeks that you are participating.

- You will need to take a malaria test every week that you are working on the study and sign a form to show that you have taken the test. The test will be paid for by the study. If you are sick we will provide you with the correct medicine to treat malaria: ALU (Artemisinin lufantrine) free of charge, and you will no longer allowed to take part in the study because you are sick.

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may change your mind later and stop participating even if you agreed earlier. This will not affect your work with IHI. It is your choice and all of your rights will still be respected.

Risks

The risk of this study is you may feel uncomfortable due to mosquito bites. You will be provided with a bed net so you will not receive many bites, although the bed nets have holes in them. There is a small possibility that you may get malaria while participating in the study. Thus, if you suffer from fever, you should immediately seek for advice/assistance from the Ifakara Health Institute personnel as per the contact details given below.

Deltamethrin and permethrin, the insecticides used on the nets, has been tested before and has not been found to have any undue adverse effects in most people at the dose found on the nets. Some tingling or runny nose has been recorded in some people when nets are used for the first time when taken from its package. There is no cause for alarm as these effects pass within a day or two. In some people, the use of treated nets may also cause other adverse effects during the first few days of their use such as headache, numbness, and itching, sneezing, discharge from eyes, nausea, and unpleasant smell. We will ask you for these symptoms, as well as should you perceive any adverse effects of using the nets, please consult a doctor at the local health facility or report this to one of our staff immediately at the contact details given below and we will provide you with all the necessary medical care.

Benefits

If you participate in this research, you will have the following benefits: You will be given weekly screening for malaria and treatment if you have parasites. You will also be helping the development of better bed nets that people like to use by providing information on the effectiveness of new products.

Compensation

You will receive Tshs 15,000 for your time away from home each night of the experiment.

Who to contact

This proposal has been reviewed and approved by the Ethics Committee of IHI and NIMR, Tanzania, which is a committee whose task it is to make sure that research participants are protected from harm.

In case you have any question or concern about this study please feel free to contact Mr. Jeremiah J Musa, Study Investigator (Tel: +255 789 000 916) and Dr. Sarah Moore, Senior Research Scientist/Study Director (Tel. No. +255 764 802 622) at IHI.

However, if you are not satisfied with responses given by the study team, feel free to contact the representative of IHI institutional review board Dr. Mrisho Mwifadhi, (Telephone: +255 788 766 676), or Ms. Sia Malekia, (Telephone: +255 754 499 293) National Institute of Medical Research (NIMR).

We are leaving with you a copy of this informed consent form for your information and future reference.

PART II: Certificate of Consent

I ……. clearly understand the aims of the project entitled

"Phase II Bio-efficacy evaluation of Long-Lasting Insecticidal nets after five years of storage (DawaPlus® 2.0 LN and Olyset®) against strongly pyrethroid resistant *Anopheles arabiensis* and fully pyrethroid susceptible *Anopheles gambiae* s.s. in the Ifakara Ambient Chamber test, Tanzania" and I agree to participate in the study. During my participation in these studies, I understand that working at night may expose me to increased risk of infection with malaria. I therefore undertake to submit to weekly screening for malaria parasites by RDT. I also understand that I am entitled to free malaria prophylaxis and treatment for malaria if found to be infected with malaria parasites. I understand that I may revoke my consent and leave the study at any time.

Participant Name: _____

Participant Signature: _____Date _____DD/MM/YY

Witness Name: _____

Witness signature: _____Date_____ DD/MM/YY)

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

*Print name of witness*_____ *AND Thumb print of participant*

Signature of witness _____

Date _____DD/MM/YY)

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. The participant has been requested to sleep under a bed net between 21:00 hrs and 06:30 hrs.

2. The participant has been informed that work will be done in the screen house tunnel so the mosquitoes do not have malaria cannot transmit disease even if they bite participants.

3. The participant has been requested to sleep in a compartment in the semi-field tunnel. Mosquitoes will be released into the compartment.

4. The participant has been informed that the bed nets are safe for use on humans and has been approved by the Tropical Pesticide Research Institute.

5. The participant has been requested to collect the mosquitoes in the compartment at 06.30 hours each morning using a siphon.

6. Participant has been requested to refrain from smoking and consuming alcohol for the study duration.

7. Participant has been requested to take a malaria test every week that they are working on the study and sign a form to show that they have taken the test

8. Participant has been informed that malaria testing and treatment will be paid for by the study.

9. Participant has been informed that if they test positive for malaria, they will not be allowed to take part in the study.

10. Participant will be reimbursed 15,000 Tshs per night for work time taken up by the study

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

I confirm that a copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____DD/MM/YYYY