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COMPARISON OF CONE BIOASSAY ESTIMATES AT TWO LABORATORIES WITH DIFFERENT *ANOPHELES* MOSQUITOES FOR QUALITY ASSURANCE OF PYRETHROID INSECTICIDE-TREATED NETS

Stephen Gabriel Mbwambo

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

Arusha, Tanzania

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ABSTRACT

This study explored utility of cone bioassays for pre-delivery quality assurance (QA) of pyrethroid insecticide-treated nets (ITNs) to test the assumption that cone bioassays are consistent across locations, mosquito strains, and laboratories. Double-blinded bioassays were conducted on 20 pyrethroid ITNs of four brands (100 nets, 5 subsamples per net) that had been delivered for mass distribution in Papua New Guinea (PNG) having passed pre-delivery inspections. Cone bioassays were performed on the same net pieces following World Health Organization (WHO) guidelines at the PNG Institute of Medical Research (PNGIMR) using pyrethroid susceptible *Anopheles farauti sensu stricto* and at Ifakara Health Institute (IHI), Tanzania using pyrethroid susceptible *Anopheles gambiae sensu stricto*. Results from IHI and PNGIMR were compared using Spearman's Rank correlation, Bland-Altman (BA) analysis and analysis of agreement. In cone bioassays, 13/20 nets (65%) at IHI and 8/20 (40%) at PNGIMR met WHO bio-efficacy criteria. Results from IHI and PNGIMR correlated on 60-minute knockdown (KD60) (r_s= 0.6, p= 0.002, n=20) and 24-hour mortality (M24) (r_s=0.9, p<0.0001, n=20) but BA showed systematic bias between the results. The agreement between the results to predict ITN failure was good with kappa=0.79 (0.53-1.00) and 90% accuracy. Based on these study findings, the WHO cone bioassay is a reproducible bioassay for ITNs with >80% M24, and for all ITNs provided inherent stochastic variation and systematic bias are accounted for. The 80% mortality (M24) threshold remains the most reliable indicator of pyrethroid ITN quality using pyrethroid susceptible mosquitoes. In the absence of alternative tests, cone bioassays could be used as part of pre-delivery QA.

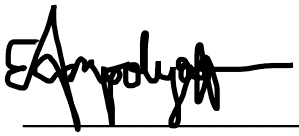
DECLARATION

I, **Stephen Gabriel Mbwambo**, do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work, and that it has not been submitted for consideration of a similar degree award in any other University.

Stephen G. Mbwambo — **Date** 03.08.2022

This dissertation has been submitted with our approval of the University Supervisors:

Prof. Sarah J. Moore — **Date** 03.08.2022
Ifakara Health Institute



Dr. Emmanuel Mpolya — **Date** 03.08.2022
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CERTIFICATION

The undersigned certifies that they have read and hereby recommend for acceptance by the Nelson Mandela Institution of Science and Technology a dissertation titled "**Comparison of Cone Bioassay Estimates at two Laboratories with Different *Anopheles* Mosquitoes for Quality Assurance of Pyrethroid Insecticide-Treated Nets**" in fulfillment of the requirements for the Degree of Master of Science in Public Health Research at the Nelson Mandela African Institution of Science and Technology Arusha, Tanzania.

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Prof. Sarah J. Moore
(Principal Supervisor)
03.08.2022

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DEDICATION

I dedicate this work to my lovely God, wife and children for being there for me, never leaving me and always loving me.

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LIST OFABBREVIATIONS AND SYMBOLS

°C	Centigrade
°	Degree
®	Registered
AI	Active ingredients
CI	Confidence Interval
IHI	Ifakara Health Institute
IMR	Institute of Medical Research
IRB	Institutional Review Board
ITNs	Insecticide treated nets
KD60	Mosquito Knockdown after 60-minutes
LLINs	Long Lasting Insecticidal Nets
M24	Mosquito Mortality at 24-h
NMCP	National Malaria Control Program
PMI	Presidential Malaria Initiative
s.s	Sensu stricto
QA	Quality Assurance
QC	Quality Control
UNICEF	United Nations Children's Fund
VCPTU	Vector Control Product Testing Unit
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
WHO-PQ	World Health Organization Prequalification

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

Pyrethroid insecticide-treated nets (ITNs) are among the recommended public health interventions for control of malaria vectors (WHO, 2021) and are estimated to have prevented more than 450 million malaria cases in Africa between 2000 and 2015 (Bhatt *et al.*, 2015). While insecticide resistance (WHO, 2018) and mosquito behavioural changes (Sherrard-Smith *et al.*, 2019) are factors contributing to the reduction of the effectiveness of pyrethroid ITNs, they can still provide a high degree of protection (Kleinschmidt *et al.*, 2018), especially in areas where *Anopheles* mosquitoes are still susceptible to pyrethroids like in Papua New Guinea (PNG) (Koimbu *et al.*, 2018; WHO, 2018) and some parts of East Africa such as west Tanzania (Hancock *et al.*, 2020).

It is important to deliver effective ITNs to protect those at risk against mosquito bites and malaria. To guarantee the effectiveness of ITNs distributed in malaria-endemic countries, it is necessary to conduct independent pre-delivery quality assurance (QA) and post-delivery operational monitoring of ITN quality (Lindsay *et al.*, 2021). Pre-delivery, ITN product specifications are checked including ITN insecticide content. Post-delivery, ITN insecticide content, bioefficacy, physical integrity, and ITN survivorship are metrics used for ITN quality monitoring (WHO, 2013a). Bioefficacy is a measurement of the ability of the ITN product to induce mortality, knockdown (sublethal incapacitation) or prevent blood feeding of mosquitoes under laboratory conditions. Minimum bioefficacy thresholds for laboratory assays (WHO, 2013a), have been set at a level measured in experimental hut trials (Miller *et al.*, 1991) that corresponded with malaria control, estimated by clinical trials conducted in Africa when mosquito vectors were still susceptible to pyrethroids (Alonso *et al.*, 1991).

Pyrethroid ITN bioefficacy is evaluated experimentally under laboratory conditions with susceptible malaria vectors using cone bioassay and tunnel tests (WHO, 2013a). Bioefficacy evaluations provide reassurance of likely impact against susceptible vectors. New or prior to use pyrethroid ITNs should meet World Health Organization (WHO) standard bioefficacy criteria, i.e.,

≥95% mosquitoes knockdown at 60-minutes (KD60) and/or ≥80% mortality at 24-hours (M24) for cone bioassays (WHO, 2013a). It has been shown by many studies that new or prior to use pyrethroid ITNs generally exhibit 100% for both or either of these bioefficacy endpoint(s) (Abílio *et al.*, 2015; Bhatt *et al.*, 2012; Castellanos *et al.*, 2021; Graham *et al.*, 2005; Kayedi *et al.*, 2007; Ketoh *et al.*, 2018; Malima *et al.*, 2013; Okia *et al.*, 2013; Rafinejad *et al.*, 2008; Tungu *et al.*, 2021; WHO, 2009a, 2015). The utility of cone bioassays is that they can estimate small variations in insecticide (Graham *et al.*, 2005) and bioefficacy (Gimnig *et al.*, 2005) that can inform the effectiveness of the intervention. For pyrethroid ITNs unable to meet cone bioefficacy criteria, a second evaluation is conducted, using the WHO tunnel test that is designed for the evaluation of ITNs treated with insecticides that have an excito-repellent mode-of-action e.g. permethrin or etofenprox (WHOPES, 2005). However, in reality tunnel tests are used for all ITNs regardless of the mode of action of the active ingredient. The performance thresholds for WHO tunnel tests are to induce ≥90% blood feeding inhibition (BFI) at 12-hours and/or ≥80% M24 (WHO, 2013a).

Physiochemical tests are currently used for ITN QA (Global Fund, 2018) on the assumption that product performance is predictable based on the product specifications measured in predelivery inspections. Available evidence indicates that the vast majority of ITNs are likely to contain sufficient insecticide when they are delivered to households (LLP, 2021). While this is encouraging, it should be remembered that predelivery inspections measure the total chemical content of the net yarn, while mosquitoes landing on the netting are exposed only to the insecticide present on the surface. The bioefficacy endpoints of KD60 or M24 are sensitive to small changes in insecticide surface concentration, which can be different between, and sometimes within, products and can be subject to change in particular when ITNs are exposed to heat (Bubun *et al.*, 2021; WHO, 2008). It has been shown that total insecticide content does not always correlate with bioefficacy (Karl *et al.*, 2021).

Differences in ITN bioefficacy may be due to variations in spatial presentation and/or distribution of active ingredient within the netting, or the surface treatment as part of the manufacturing process. ITNs are manufactured from polyester or polyethylene, and careful product design and quality controlled manufacturing is required to ensure adequate bioavailability of active ingredient over the life of the product (WHO, 2008). However, chemical assays of surface concentration,

such as the cyanopyrethroid field test and chemical tests such as high performance liquid chromatography and gas chromatography have not yet been found to correspond well to bioefficacy results.

1.2 Statement of the problem

Nets prequalified and listed by WHO are manufactured accordance with product specifications. For manufacturers to meet donor funding and procurement policy should exhibit quality control tests optimal criteria including safety, efficacy and quality (Global Fund, 2018). While quality control tests for product specifications are encouraging to protect health of individuals against malaria vectors, not all tests are currently used to monitor product performance (LLP, 2021). Recently, recipient countries including Tanzania and PNG conduct predelivery quality assurance on ITNs but mostly encompass physio-chemical but not bioefficacy tests (LLP, 2021). However, it should be remembered that predelivery inspections measure the total chemical content of the net yarn, while mosquitoes landing on the netting are exposed only to the insecticide present on the surface. For instance, samples of new PermaNet® 2.0 nets delivered in PNG between 2013 and 2019 chemical test revealed full insecticide content (Karl *et al.*, 2021) but the same samples on bio-efficacy tests did not kill or knockdown mosquitoes against pyrethroid susceptible mosquitoes *Anopheles farauti* (Karl *et al.*, 2021; Vinit *et al.*, 2020). Further investigation reports or studies in some endemic countries including Rwanda (Karema *et al.*, 2020), Benin (Ahogni *et al.*, 2019), Nigeria (Daniel, 2006), Cambodia (WHO, 2017), Iran (Bagheri *et al.*, 2017), Madagascar (Randriamaherijaona *et al.*, 2017) have reported delivery of sub-optimal efficacy of new (prior to use) ITNs subsequently distributed to the malaria endemic population with increasing significant number of malaria cases. Also of note, there are evidence that ITNs with low quality are available in markets in some parts of Tanzania (IPP, 2016).

It is generally agreed that a validated, low-cost, easy-to-implement laboratory methodology for assessing surface AI content is urgently needed (LLP, 2021) but current methods have not been found to correspond well to bioefficacy results. Cone bioassays have been demonstrated to be highly sensitive to changes of active ingredient concentration on the net surface, simple, cost effectiveness and could thus play a crucial role in ITN QA (WHO, 2007). This study explored utility of cone bioassays for pre-delivery QA in two test facilities using different *Anopheles* strains

to test if cone bioassays are consistent and reproducible across locations, mosquito strains, and laboratories.

1.3 Rationale of the study

The chemical tests have been used to determine the quantity of insecticide dose content in the ITN. However, the chemical test does not provide information on insecticide doses available on the surface fiber (Karl *et al.*, 2021). Previous chemical test results revealed a full insecticide concentration within nets but did not effectively kill or knockdown susceptible *Anopheles* mosquitoes as recommended by WHO (Karl *et al.*, 2021). Inclusion of bioassay test at predelivery quality assurance in recipient countries will guarantee optimal efficacy of vector control tools prior to distribution and will complement the effectiveness of the intervention.

1.4 Research Objectives

1.4.1 General Objective

To explore utility of cone bioassay for pre-delivery QA of ITNs in two test facilities using different *Anopheles* strain to test the assumption that cone bioassays are consistent and reproducible across locations, mosquito strains, and laboratories.

1.4.2 Specific Objectives

(i) To determine the bio-efficacy of the same pyrethroid ITN products (Interceptor®, PermaNet®, SafeNet®, Yorkool®) at two laboratories with different *Anopheles* strain.

(ii) To compare the cone bioassay estimates of the same pyrethroid ITN products (Interceptor®, PermaNet®, SafeNet®, Yorkool®) at two laboratories with different *Anopheles* strain.

1.5 Research Questions

(i) Do the pyrethroid ITN products (Interceptor®, PermaNet®, SafeNet®, Yorkool®) tested at two laboratories meet WHO standard bioefficacy criteria against different *Anopheles* strain?

(ii) Can two laboratories obtain reproducible results when testing the same nets against different *Anopheles* strain?

1.6 Significance of the study

This study provides crucial information to the malaria control programs and potential stakeholders that the cone bioassay test may be among of components in pre-delivery quality assurance and/or prior to distribution to control ITN bioefficacy.

1.7 Delineation of the study

The number of nets tested in this study may not be sufficient to generalize the study results. Therefore, a systematic review on the utility of cone bioassays for prior to use pyrethroid ITNs testing was also conducted and showed results in broad agreement with those reported in present study. Many publications and reports included in the systematic review did not indicate country of manufacture, ITN age, and the lot or batch numbers of the tested nets, or data collection period. Thus, it was only possible to present the date/year that the study was conducted and/or the publication date. This study was not conducted using the ideal full factorial design with the same strains in each laboratory (that would tease out species versus laboratory differences) due to biosafety concerns because both laboratories are in malaria endemic areas. It would not be safe to establish either malaria vector in the other laboratory.

CHAPTER TWO

LITERATURE REVIEW

2.1 Reliance on ITNs

A decade ago, pyrethroid ITN was innovated to safeguard people against vector-borne diseases particularly malaria. The ITN has number of public health value such as; individuals use it in the house, can protects them against vectors contact for blood feeding and other people in the same house who are not using it (Hawley *et al.,* 2003; Schellenberg *et al.*, 2001). This is expanded to the endemic population where optimal coverage of treated bed nets protects the segment of the population that is not sleeping under nets (Hawley *et al.,* 2003; Lengeler & Snow, 1996). The concept is due to community offering insecticidal effect to reduce longevity and survival of the mosquito to complete parasite life cycle (Lengeler & Snow, 1996). Pyrethroid ITNs are among the recommended public health interventions for control of malaria vectors (WHO, 2021) and are estimated to have prevented more than 450 million malaria cases in Africa between 2000 and 2015 (Bhatt *et al.*, 2015). While insecticide resistance (WHO, 2018) and mosquito behavioural changes (Sherrard-Smith *et al.*, 2019) are factors contributing to the reduction of the effectiveness of pyrethroid ITNs, they can still provide a high degree of protection (Kleinschmidt *et al.*, 2018), especially in areas where *Anopheles* mosquitoes are still susceptible to pyrethroids like in Papua New Guinea (PNG) (Koimbu *et al.*, 2018; WHO, 2018) and some parts of East Africa such as west Tanzania (Hancock *et al.*, 2020).

2.2 Prequalified and listed ITNs by WHO Prequalification team

Among of 22 ITN products pre-qualified and listed by WHO-PQ (WHO-VCP, 2020), encompass nets manufactured with different active ingredients (AI). Such as pyrethroid only nets, pyrethroid + piperonyl butoxide (synergist) nets, and combined two pyrethroid AI nets (WHO-VCP, 2020). Even so, pyrethroid nets contribute around 64% of listed nets, and most are manufactured with alpha-cypermethrin and deltamethrin AI (UNICEF, 2020). The dissimilar AI in the nets may have variations in insecticidal activity or mode of action. The insecticidal activity in the bed net depends on the type of active ingredient impregnated/coated on or incorporated within a net, amount of dose or concentration of the insecticide, sub-lethal dose on the surface of the net, and state or

characteristics of insects (Lengeler & Snow, 1996).The current study deployed prior to use nets impregnated with alpha-cypermethrin or deltamethrin insecticides. The nets classified into two: (a) new nets of different brands and batch manufactured between 2019 and 2020 retained in PNGIMR warehouse under control storage conditions before distributed to the entire population of PNG and (b) old nets of the same brand stored under tropical temperature and relative humidity for 6 years and retain in controlled storage conditions. It was deemed necessary to have sample of nets from Tanzania, because even the net brands were distributed in PNG also are currently distributed in Tanzania population through school net programme. However, was not possible to include the samples from Tanzania because there was absence of country investigation reports that low quality nets distributed to the malaria endemic population (Table 4). Although has been reported low quality nets in markets at some parts of Tanzania (IPP, 2016). Design of this study was to see if the same nets performed poor after distributed to the entire population in PNG (Vinit *et al.*, 2020) will perform better or reveal the same results against WHO reference testing strain mosquitoes in Tanzania i.e susceptible *Anopheles gambiae s.s* and assessing the bioassay tests at two laboratories if provide consistent and reproducible bioassay estimates against different mosquito strains and other assay parameters not specified by the current WHO guidelines.

2.3 Competitive market of ITNs

The majority of funding sources for procuring ITNs to the majority of endemic countries are donors, international agencies, development partners such as the Global Fund, UNICEF, USAID/PMI, or the World Bank (Global Fund, 2019), and some of governments. These partners are purchasing ITNs instead of the demanded countries. The reason is highly expensive for the government or individuals to afford LLIN unit cost or price (WHO, 2004a). Also, at started LLIN technology there were limited numbers of LLIN manufacturers to satisfy demand (WHO, 2004a). At least 2006 has been increased number of LLIN manufacturers. For example, A-Z Company producing Olyset® net transfer technology from Sumitomo introduced it in Arusha Tanzania (WHO-VCP, 2020). Currently, LLINs manufacturing company increased from 2 in 2004 to 12 in 2020 (UNICEF, 2020) and almost half (250 million) of produced nets (400 million) annually are delivered to the endemic countries (UNICEF, 2020). Resulted more than half decline of LLIN unit cost for at least 10 years from 6 USD in 2008 (Wafula *et al.*, 2013) to 1.88 USD in 2019 (UNICEF,

2020) may be affecting the suppliers to produce effective products and manage with market dynamic strategy.

2.4 The quality control and quality assurance of ITNs

The quality control practice of ITN may require taking place at any stage from the production to post-distribution, because of an accidental change of master batch, less heat stable and other disagreement processes may yield poor quality or lower efficacy nets. For this reason, quality assurance and/or monitoring studies are conducted following these processes and involves manufacturers, suppliers and other stakeholders such as WHO-PQ, the Global Fund and purchasers (ALMA, 2012). Outcomes are interpreted accordance with agreed pre-defined parameters and its minimum performance indicators. However, in binding process if pre-shipment inspection providing confidently on LLIN quality will not require pre-delivery inspection in recipient countries (ALMA, 2012; Global Fund, 2019). Recently, recipient countries conduct pre-delivery quality assurance on ITNs but mostly encompass physio-chemical but not bioefficacy tests (LLP, 2021).

2.5 Testing ITNs bioefficacy

The ITN efficacy is routinely evaluated at initial tests under laboratory conditions using susceptible malaria vectors and semi-field whereby wild mosquitoes are exposed in the designed tent in presence of human bait (WHO, 2013a). The WHO recommended cone and tunnel bioassay tests to be used on ITNs bioefficacy evaluation under laboratory conditions (WHO, 2013a). For pyrethroid ITNs unable to meet cone bioefficacy criteria (≥95% KD60 and/or ≥80% M24), a second evaluation is conducted using the WHO tunnel test that is designed for the evaluation of ITNs treated with insecticides that have an excito-repellent mode-of-action e.g. permethrin or etofenprox (WHOPES, 2005). However, in reality tunnel tests are used for all ITNs regardless of the mode of action of the active ingredient. The performance thresholds for WHO tunnel tests are to induce ≥90% blood feeding inhibition (BFI) and/or ≥80% M24 (WHO, 2013a). Moreover, a tunnel tests has not been established to other testing facilities may be because for long period ago a pyrethroid nets was a strong recommended vector control tool thus need only cone bioassay for testing under laboratory conditions. Even so, current study in Tanzania has been shown that

establishment of a tunnel test in testing facility needs more investment and resources to run an insectary and baits (Kamande *et al.*, 2022). The absence of additional test in testing facilities may led bias and inability to compare ITN bioefficacy results among intra and inter laboratories. For instances the quality assurance study or routine testing nets in cone bioassay should consist further confirmation test with longer mosquitoes' exposure such as tunnel tests or semi field.

2.6 Cone bioassay test for pre-delivery quality assurance

The best choice of quality assurance test for investigating bioefficacy at pre-delivery or/and prior to distribution required to meet characteristics such as low-cost to operate, simple, accurate, reliable and reproducible test data (LLP, 2021; Rafinejad *et al.*, 2008; Vontas *et al.*, 2014; WHO, 2010). The cone bioassay test may be met the choice criteria mentioned above. It is known that cone bioassay results can be affected by ITN characteristics i.e. manufacturing, poor shipping (AMP, 2020) or storage conditions as well as bioassay methods including sample preparation e.g. using a net sample straight from the fridge (Skovmand, personal communication), mosquito age (Kulma *et al.*, 2013; Marti-Soler *et al.*, 2021) and fitness (Owusu *et al.*, 2017), test procedures (Owusu & Müller, 2016), temperature (Glunt *et al.*, 2018; Glunt *et al.*, 2014; Hodjati & Curtis, 1999a) and inter-operator variability (WHO, 2008). However, not much is known about whether cone bioassay results are susceptible to systematic bias and random variability depending on the *Anopheles* strain used and other assay parameters not specified by the current WHO guidelines.

2.7 Systematic review of cone bioassays for QA of pyrethroid ITNs

To get at the question of whether cone bioassays might be a viable method for assessing bioefficacy prior to ITN distribution and as the current study comprises a limited number of nets it was deemed necessary to conduct a systematic review on cone bioassay for bioefficacy evaluation of prior to use pyrethroid nets. The aim of this review was to investigate how frequently WHO cone bioassays are used to test new, unwashed pyrethroid ITNs and whether cone bioassays are considered a suitable method for this purpose. A search of the literature on ITN efficacy studies, durability studies or WHOPES specification reports published between 2001 and 2021 was conducted in October, 2021 in PubMed and PubMed Central using the keywords "bio-efficacy" or "cone bioassay tests" and "tunnel tests" or "Insecticide treated nets" and "long lasting insecticidal nets"

and Google Scholar using the keyword "WHOPES working group meeting". Overall, the literature search identified 2362 titles (PubMed: 87 titles, PubMed Central: 1604 titles and Google Scholar: 671 titles). Titles were further screened for reports using standard WHO evaluation methods on prior to use pyrethroid ITNs with *Anopheles* mosquitoes that reported both KD60 and M24. This resulted in seventy publications being fully screened and sixty being included in the final selection. Data extracted from selected publications included ITN type (brand name, active ingredient, manufacturing technology, manufacturing date or year, batch/lot number), bioassay results (mainly KD60 and M24), the *Anopheles* strain used in the bioassays and where and when the study was conducted.

The systematic review on the use of WHO cone bioassays for pyrethroid ITN testing showed that the vast majority of prior to use pyrethroid ITNs scored high KD60 and M24 (Fig. 1). On average KD60 was 96% (95% CI: 94-98) and M24 was 92% (95% CI: 88-96). From the 83 observations with unwashed ITNs that included both KD60 and M24 observations, mainly with *An. gambiae* s.s (63/83) and mainly with deltamethrin coated ITNs (51/83) only 12 reported KD60 below 95% (Table 4). Interestingly, even permethrin ITNs gave very high knockdown 89% (95% CI: 74-100) and mortality 89% (95% CI: 68-100) in studies published between 2008 and 2017.

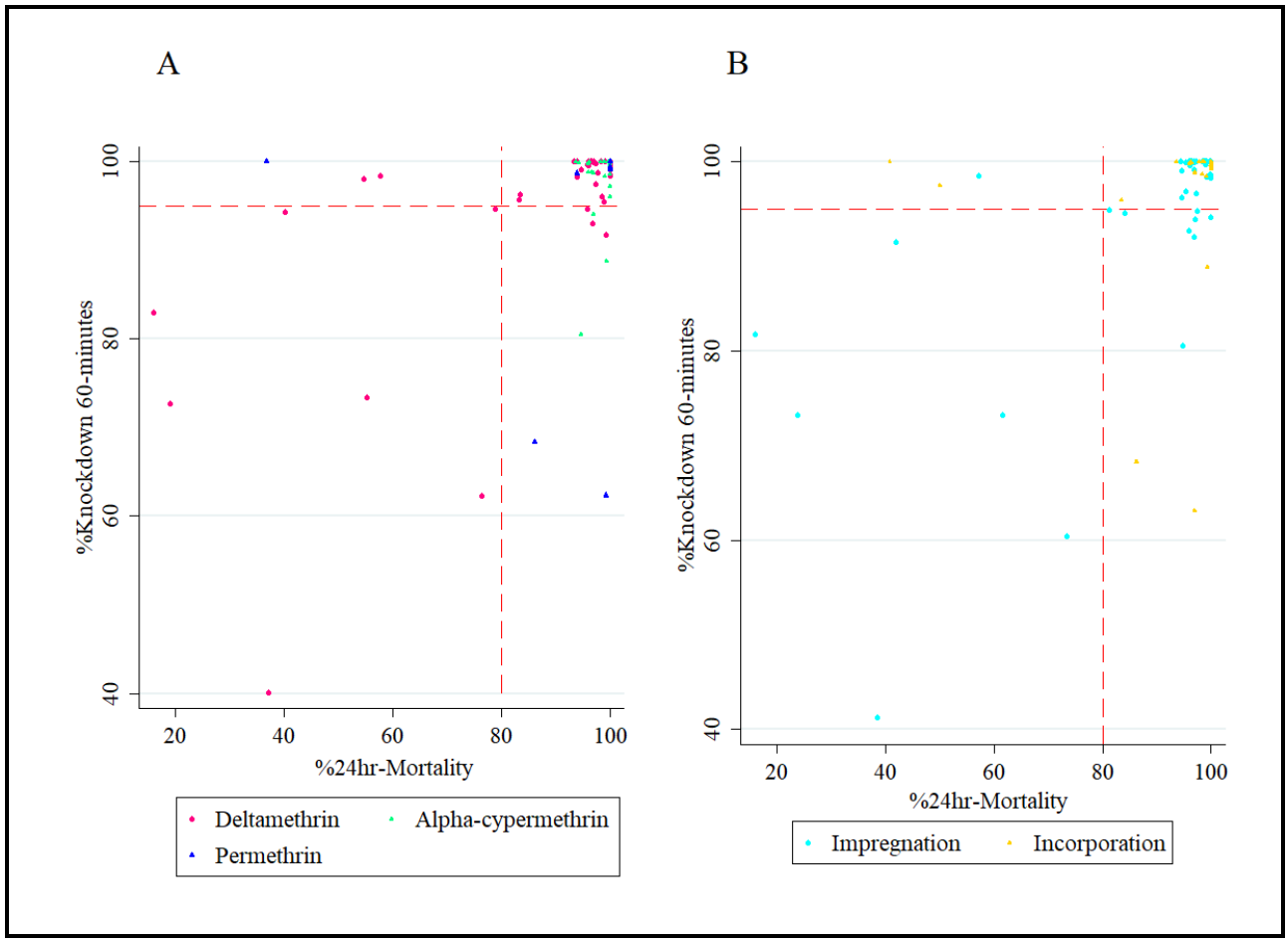

Figure 1: Results from the systematic review

(**A)** Relationship between KD60 and M24 in WHO cone bioassays with pyrethroid ITNs (deltamethrin, alpha-cypermethrin and permethrin) using *Anopheles* mosquitoes. (**B)** Relationship between KD60 and M24 in ITNs grouped by production technology. Dashed lines are the WHO threshold 95% KD60 and 80% M24.

This study explored utility of cone bioassay for pre-delivery QA in two test facilities using different *Anopheles* strain to test the assumption that cone bioassays are consistent and reproducible across locations, mosquito strains, and laboratories.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

A double-blinded comparison of pyrethroid ITN bioefficacy as measured by WHO cone bioassay was conducted in two testing facilities. 20 pyrethroid ITNs of four brands (100 subsamples, i.e., five subsamples per net) that had passed predelivery inspections were assessed under laboratory conditions following WHO guidelines (WHO, 2013a). ITN subsamples were first evaluated using WHO cone bioassays and those that did not meet the WHO cone bioassay performance criteria (≥95% KD60 or ≥80% 24-hours mortality) were tested using the WHO tunnel test at IHI following standard procedures (WHO, 2013a).

3.2 Testing facilities

The experiments were conducted at the Vector-borne Diseases Unit (VBDU) of the Papua New Guinea Institute of Medical Research (PNGIMR) and the Vector Control Product Testing Unit (VCPTU) of the Ifakara Health Institute (IHI) in Tanzania that is Good Laboratory Practice (GLP) accredited, South African National Accreditation System (SANAS) G0033 (SANAS, 2021). This study was conducted in partnership between IHI, Tanzania and PNGIMR because:

(i) In PNG, investigated that samples of new PermaNet® 2.0 nets delivered in a country between 2013 and 2019 subsequently distributed to the malaria endemic population were ineffectiveness against the local strains of pyrethroid susceptible mosquitoes *Anopheles farauti* (Vinit *et al.*, 2020).

(ii) In PNG, the principal malaria vectors are *Anopheles farauti*, *Anopheles koliensis* and *Anopheles punctulatus.* WHO bioassay laboratory study recommended minimum performance susceptible *Anopheles gambiae* a reference strain known as biological parameters test system (WHO, 2001). For this reason, if the nets kill susceptible *An. gambiae* (available in IHI, Tanzania) there is high chance that nets will kill other susceptible *Anopheles* strains. Therefore, we guarantee the use of *Anopheles gambiae*-a

local strain in Tanzania has provided more justification on laboratory results against the same nets tested at PNG against *Anopheles farauti.*

(iii) A recent 'Landscaping of ITN Bioefficacy Report for the Global Fund recommended a Institutions/testing facilities partnership for conducting a bioefficacy study on same net samples to assess if standard bioassay tests are consistent and reproducible across locations, mosquito strains, and laboratories for ITN quality monitoring both pre-and post-shipment (LLP, 2021).

Table 1: Summary of experiments

Experiment	***Bioassay test in IHI***	***Cone bioassay test in PNGIMR***
Number of ITNs tested	20 nets (100 net pieces)	20 nets (100 net pieces)
Mosquitoes exposed	20 per net piece (cone bioassay) 100 per net piece (tunnel tests)	20 per net piece
Experiment conditions	27±1°C 55% - 82% relative humidity	28 ±4°C 53%-71% relative humidity
Mosquito species	Pyrethroid susceptible[*] *An. gambiae* s.s	Pyrethroid susceptible[*] *An. farauti* s.s
Mosquito age	3-5 days (cone bioassay) 5-8 days (tunnel tests)	2-5 days
WHO efficacy criteria	≥ 95% KD60 or ≥80% M24 (cone bioassay) ≥ 90% FI and/or ≥80% M24 (tunnel tests)	≥ 95% KD60 or ≥80% M24

[*]Sugar fed *Anopheles gambiae* s.s (Ifakara) *and Anopheles farauti* s.s were confirmed to be 100% susceptible to alpha-cypermethrin, deltamethrin and permethrin insecticides at 1x WHO discriminating concentration at the time of evaluation: KD60 knockdown measured at 60 minutes (sublethal incapacitation), M24 mortality measured at 24 hours post exposure and FI feeding inhibition.

3.3 Description of tested products

Five products (rectangular nets) were included in the study. PermaNet® 2.0, a blue multi-filament polyester, 75 denier coated with 1.8g/kg (55mg/m^2) deltamethrin and manufactured in 2019 by Vestergaard Frandsen, in Vietnam; PermaNet® 2.0, a yellow multi-filament polyester fiber, 75 denier coated with 1.8g/kg (55mg/m^2) deltamethrin and manufactured in 2012 (manufacture location not given on label); Interceptor®, a blue multi-filament polyester fiber, 100 denier coated with 5g/kg (200mg/m^2) alpha-cypermethrin and manufactured by BASF in Thailand; SafeNet®, a blue multifilament polyester net, 100 denier coated with 5g/kg (200mg/m^2) alphacypermethrin (manufacture location not given on label); Yorkool®, a blue multifilament polyester net, 75 denier coated with 1.8g/kg (55mg/m^2) deltamethrin and manufactured by Tianjin Yorkool International Trading Company limited, China. Negative control net: untreated SafiNet® was made of polyester fibres manufactured by A to Z textile mills, Tanzania and untreated Baomei® was made of polyester fibres net manufactured in China were used in IHI and PNGIMR, respectively.

3.4 Net origin and storage condition

The PermaNet® 2.0 manufactured in 2012 (PermaNet®2012) nets were distributed in the year 2012 through the mass distribution campaign in all Regions of PNG. These ITNs in unopened packaging were stored under tropical temperature and humidity in a store room of the Madang Provincial Health Authority between 2012 and 2018. The nets were transferred to a PNGIMR store in 2018 and kept at around 27°C. PermaNet® 2.0 manufactured in 2019 (PermaNet®2019), the Interceptor® manufactured in 2020, the SafeNet® manufactured in 2019 and 2020 and the Yorkool® manufactured in 2019 were collected from shipping containers immediately upon arrival in PNG and prior to distribution, and stored a PNGIMR store room at around 27°C.

3.5 Net subsamples preparation and coding

The sampled ITNs were labeled serially from 001 to 020 at PNGIMR. From these nets, ten net piece samples (25 cm x 25 cm) were cut. Pairs of samples were cut from adjacent positions 1 to 5 as shown in (Fig. 2) (WHO, 2013a). One net piece per position per net was sent to IHI and the second, adjacent piece was retained in PNG for testing. Thus, one hundred net pieces were each tested in PNGIMR and IHI in Tanzania. The five subsamples per net were given unique codes as

A, B, C, D, and E, were wrapped individually in aluminum foil and stored in a temperature-controlled refrigerator at 4°C.

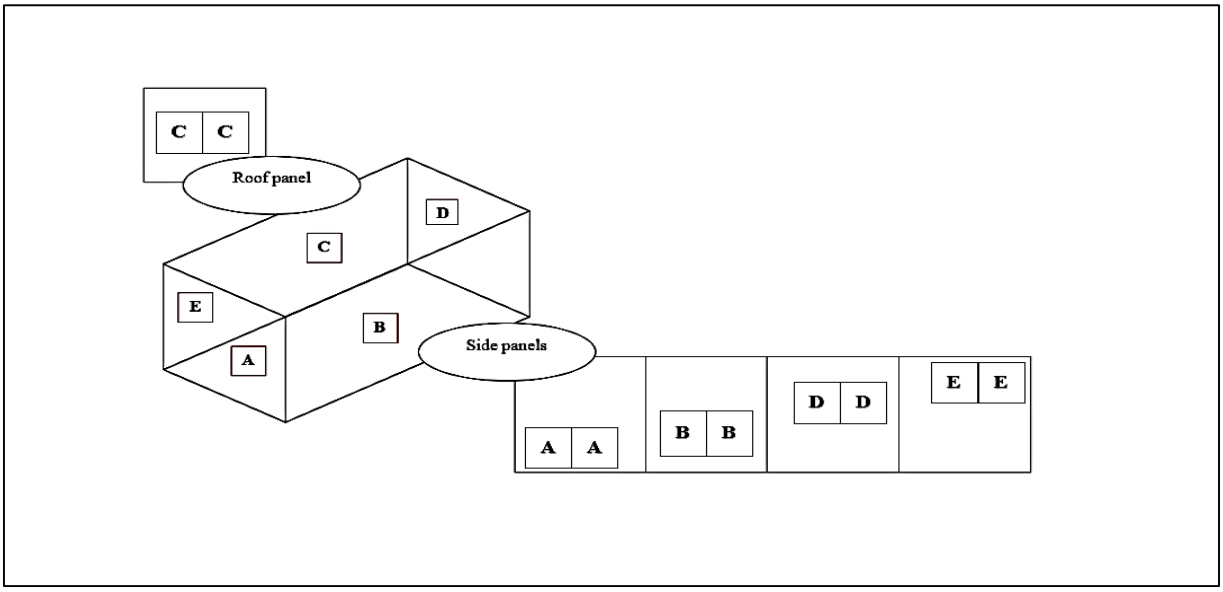

Figure 2: Rectangular whole net with five sides

As indicated in Fig. 2, net piece samples were cut from bottom side (A), middle side A (B), roof (C), middle side B (D) and top side (E). Subsamples were received in IHI in December 2020 from PNGIMR and immediately packed in new aluminum foil stored in a temperature-controlled refrigerator at 4°C. The project investigators and facility technicians were blinded and unable to identify the products until the end of the study. After all experiments were completed and data were entered, data from PNGIMR cone bioassays was sent to IHI and the blinding was disclosed to the IHI investigators to match the results from the same type of study net types to enable analysis.

3.6 Mosquito rearing and physiological status

Tanzania: Nulliparous female pyrethroid susceptible *An. gambiae* s.s. (Ifakara strain) were used; sugar fed, aged between three to five days old in cone bioassays, and sugar starved for six to eight hours, aged between five to eight days old in WHO tunnel test. The mosquito colony is maintained according to MR4 Guidelines (MR4, 2009) at 27 ± 2˚C and relative humidity of 40% - 100%, with

ambient (approximately 12:12) light dark cycle larvae were maintained on Tetramin fish flakes and adults were provided with 10% sucrose solution *ad libitum* and cow blood for egg laying.

PNG: Nulliparous female pyrethroid susceptible *An. farauti* s.s. were used; sugar fed, aged between two to five days old in cone bioassays. The colony is maintained at 28 ± 4°C and 68 ± 25% relative humidity, with approximately 11 h dark and 12 h light cycle, including a 30 min dusk and 30 min dawn period. The larvae are fed ground fish food (Marine Master Tropical Fish Flakes, Australia). The adults are provided 10% sucrose solution *ad libitum* and human blood for egg laying.

Cone bioassay procedures

On each 25cm by 25cm net piece, four standard WHO cones were fixed on a plastic cone board with holes cut and held at 60° (Owusu & Müller, 2016) at IHI, Tanzania (Fig. 3A) to maximize space and mosquito contact with the ITN, and on a board at 45º (WHO, 2013a) at PNGIMR (Fig. 3B). Net pieces were taken from the fridge and kept at room temperature for 2 hours before testing. Five laboratory-reared susceptible mosquitoes were placed in each cone for 3-minutes after which, mosquitoes were removed gently from the cones using a mouth aspirator and kept in individually labelled paper cups, one for each cone. During the holding period, mosquitoes were provided with cotton wool moistened with 10% sucrose solution. Four replicates of 5 mosquitoes were performed on each of the five net pieces making a total of 100 mosquitoes exposed per net. Endpoints measured were KD60 and M24. Mosquitoes exposed to untreated net pieces (negative controls) were tested alongside every replicate to monitor the quality of the bioassay. The bioassays and holding period were carried out at 27± 1°C and at 55% - 82% relative humidity in Tanzania and 28± 4°C and at 53% - 71% relative humidity in PNG. If the M24 exceeded 10% in a negative control, the test was repeated and if the mortality in a negative control was equal or below 10%, the results were adjusted using 'Abbott's formula' (WHO, 2013a).

A B

Figure 3: WHO cones fixed on plastic cone board

Tunnel test procedures

WHO tunnel tests were only performed in IHI Tanzania because tunnel tests are not currently established at PNGIMR. Two out of five subsamples of nets that did not meet the WHO cone bioassay efficacy criteria, were selected for the WHO tunnel test against susceptible *An. gambiae s.s.* as per WHO guidelines, these were the subsamples that gave mortality closest to the average mortality in the cone bioassay. Tunnel tests were conducted following WHO guidelines (WHO, 2013a). Non-blood fed nulliparous females 5-8 days old, sugar starved for 6-8 hours were released in a tunnel made of glass, 60 cm length. At each end of the tunnel, a 25-cm square mosquito cage covered with polyester netting was fitted. At one third of the length, a 25 cm x 25 cm swatch of netting sample was affixed. The surface of netting "available" to mosquitoes is 400 cm^2 (20 cm x 20 cm), with 9 x 1 cm in diameter holes: one hole was located at the centre of the square; the other eight were equidistant and located at 5 cm from the border. In the shorter section of the tunnel, a small rabbit shaved on its back and restrained in a mesh tunnel was placed as bait. Each rabbit was rested for more than three days after use as a bait to ensure welfare. In the cage at the end of the longer section of the tunnel, 100 female mosquitoes were introduced at 21:00 hours. The following morning at 09:00 hours, the mosquitoes were removed using a mouth aspirator and counted separately from each section of the tunnel, and mortality and blood feeding rates were

recorded. The mosquitoes were placed in paper cups and provided with cotton wool moistened with 10% sugar solution. Mortality (M24) was recorded at around 09:00 hours the following day. Mosquitoes exposed to untreated net pieces were used as controls to monitor the quality of the bioassay. The bioassays and holding period were carried out at 27º C ± 2ºC and 60% - 100% relative humidity. Overall mortality was measured by pooling the mortalities of mosquitoes from the two sections of the tunnel. Acceptable feeding success and M24 in controls were >50% and <10%, respectively. Any tests that did not achieve the specified control cut off were repeated, all results were adjusted for control mortality using Abbott's formula (WHO, 2013a).

3.7 Sample size

The sample size of four nets per tested product was based on WHO guidelines (WHO, 2013a) for testing ITNs. Post hoc power analysis of Cohen's kappa indicated there was 90% statistical power to detect a difference of up to 20% between facilities (Kappa, 2022).

3.8 Statistical analyses

Paper data collection sheets were used to record data, which were double-entered in Microsoft Excel®. Data were analysed using Stata® statistical package version 14 (Stata Statistical Software: Release 14. College Station, TX: StataCorp). Proportional KD60 and M24, or BFI and M24 were presented as arithmetic means with their respective 95% confidence intervals (CI). Pass or fail for each net was calculated based on WHO standard efficacy criteria i.e. ≥ 95% KD60 and/or ≥80% M24 for cone assay; ≥90% BFI and/or ≥80% M24 for WHO tunnel test. However, we also considered that the 80% M24 and 95% KD60 thresholds in WHO cone bioassays are subject to stochastic variation. If tests are done using 100 mosquitoes per net as per WHO guidelines, we expect an assay-inherent 95% CI of 71% and 87% around the 80% mortality threshold and a 95% CI of 89% and 98% around the 95% KD60 threshold. ITNs with a mean below the bioefficacy threshold but with 95% CIs that exceeded the bioefficacy threshold of 95% KD60 or 80% M24 were also categorized as pass.

The Spearman rank correlation coefficient (r_s) was calculated to estimate the degree of correlation between IHI and PNGIMR cone bioassay results for KD60 and M24. Bland-Altman methods (Bland & Altman, 1999) were used to assess the agreement between individual measurements of

KD60 and M24 from IHI and PNGIMR testing facilities. Cohen's kappa (κ) was used to assess the degree of agreement between facilities to predict if nets passed or failed WHO cone bioassay threshold criteria.

3.9 Ethics approval and consent to participate

Approval for the study was provided by the Institutional Review board of Ifakara Health Institute (IHI/IRB/No: 23-202), PNGIMR Internal Review Board and the PNG Medical Research Advisory Committee (MRAC 21.02). This study did not involve humans as study participants. Rabbits used in the WHO tunnel tests are cared under veterinary supervision and were given due care as per standard practices, accredited by SANAS.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.2 Bioefficacy of tested ITNs at two laboratories

At IHI, Tanzania, 13/20 nets (65%) met the WHO cone bioassay bioefficacy criteria of ≥95% KD60 and/or ≥80% M24. The seven nets that did not meet cone bioassay criteria, met bioefficacy criteria of ≥90% BFI and ≥80% M24 in the WHO tunnel tests. At PNGIMR, 8/20 nets (40%) met WHO cone bioassay bioefficacy criteria.

Table 2: WHO cone bioassay and tunnel test results

Test Item	Cone test			Tunnel test*		#Nets pass combined cone and tunnel tests
	%KD60 (95%Cl)	%24-h Mortality (95%Cl)	#Nets pass in cone	%Feeding inhibition (95% Cl)	%24-h Mortality (95% Cl)	
IHI						
PermaNet®+	100	99.7 (99.2-100)	4/4			4/4
PermaNet®++	80.0 (76.0-84.0)	22.3 (17.8-26.7)	1/4	98.3 (94.7-100)	97.8 (94.5-100)	4/4
Interceptor®	85.8 (82.6-88.9)	37.9 (32.0-43.7)	1/4	99.7 (99-100)	99.5 (98.5-100)	4/4
SafeNet®	97.3 (95.6-98.9)	61.1 (55.2-67.0)	3/4	100	100	4/4
Yorkool®	96.8 (94.8-98.7)	59.7 (56.1-63.3)	4/4			4/4
SafiNet®[a]	0	5		100[**]	5	
PNGIMR						
PermaNet®+	96.4 (92.3-100)	99.6 (98.8-100)	4/4			
PermaNet®++	37.1 (29.3-44.9)	25.9 (14.1-37.6)	0/4			
Interceptor®	79.3 (72.7-85.8)	72.8 (66.7-78.8)	0/4			
SafeNet®	82.0 (75.1-88.9)	81.0 (74.8-87.2)	1/4			
Yorkool®	87.3 (83.5-91.0)	88.5 (83.9-93.1)	3/4			
Boamei®[a]	0	5				

[*] Tunnel test performed to the nets that did not meet optimal efficacy criteria in cone bioassay (≥95% KD60 and/or ≥80% M24) at IHI. The tests are not currently established at PNGIMR.
[+] PermaNet® 2.0 manufactured in 2012,
[++]PermaNet® 2.0 manufactured in 2019.
[**]Feeding success by susceptible *Anopheles* mosquitoes in a tunnel tests
[a] Nets without active ingredients (control)

4.3 Level of correlation between IHI and PNGIMR on cone bioassay results

Correlation between IHI and PNGIMR results was statistically highly significant but with a stronger association between M24 results (r=0.9, p<0.0001, n=20) than between KD60 results (r=0.6, p=0.002, n=20) (Fig. 4). While there was some discrepancy in results on KD60 and M24, the bioassay was predictive of pass or failure. Those nets that failed WHO bioefficacy criteria in IHI also failed in PNGIMR except for two nets (5 and 12). PermaNet®2012 ITNs exceeded thresholds of KD60 or M24 in both facilities i.e., those nets passed WHO bioefficacy criteria (KD60 or M24) in IHI also passed in PNGIMR with the exception of net 2 (Fig. 4). Overall results show a higher knockdown rate and lower mortality rate at IHI relative to PNGIMR.

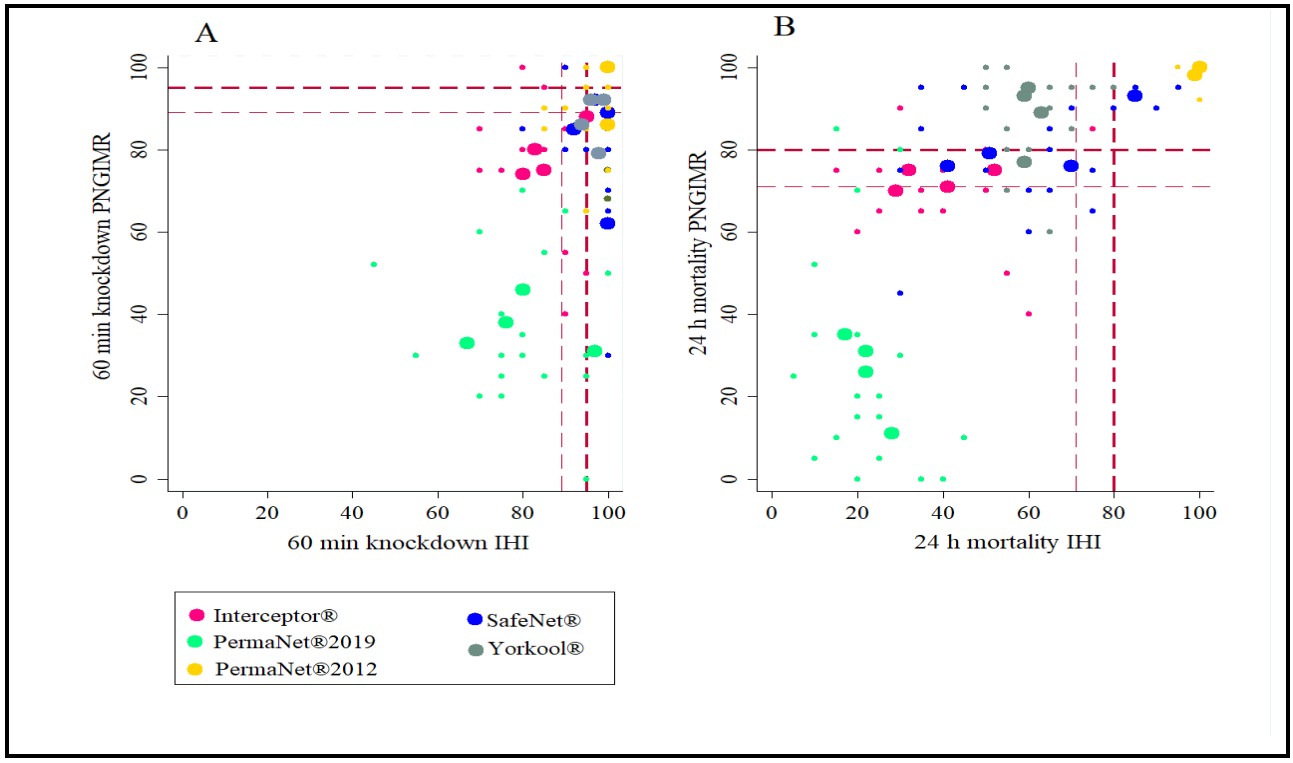

Figure 4: Correlation of cone bioassay estimates at two laboratories

As indicated in Fig. 4, thick dashed lines are the WHO threshold 95% KD60 (A) and 80% M24 (B). Thin dashed lines indicate these assay-inherent 95% (lower) CIs of these thresholds. Large dots represent averages per sampled nets (4 per net type) and small dots represent all subsamples (5 per net).

4.4 Agreement of cone bioassay at two laboratories based on KD60 and M24

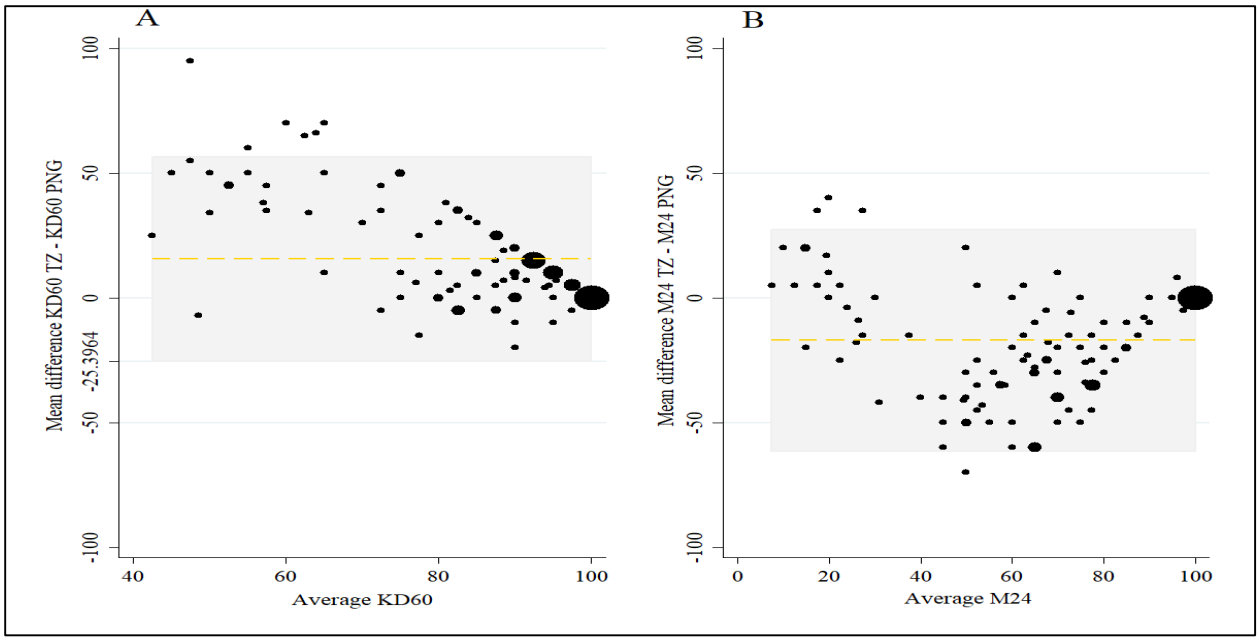

Figure 5: Bland-Altman Plots

As indicated in Fig. 5, the mean difference (y axis) plotted against the average value from both sites (A) KD60 and (B) M24. For KD60 Mean difference (limits of agreement) was 15.5 (-25.4 to 56.5) and for M24 Mean difference (limits of agreement) was -17.0 (-61.4 to 27.3). At lower mean values of knockdown, the agreement between the two testing facilities was lower than at higher mean values of knockdown but there was a consistent difference in mean difference in M24 measured at each testing facility.

4.5 Agreement of cone bioassay at two laboratories based on WHO pass/fail criteria

To account for variability inherent to the cone bioassay, the mortality pass rate was set at 71% and the KD60 rate to 89% (i.e., the lower 95% CIs of each). Thereafter, IHI and PNGIMR data agreed for n=18 (90%) of the ITNs (based on combined estimate from 5 net pieces), classifying n=6 (30%) as “fail” at both facilities and n=12 (60%) as “pass” at both facilities (Table 3). Also of note, of the five nets that demonstrated discordant pass or fail between facilities using the standard WHO bioefficacy thresholds (ignoring variability), three ITNs were re-categorized as pass in PNGIMR using the revised threshold. These nets had passed on KD60 at IHI and although failed both

bioefficacy criteria at PNGIMR but their 95% confidence interval overlapped the optimal bioefficacy threshold of 80% M24 (Fig. 6).

The agreement between the bioefficacy results at IHI and PNGMR to predict ITN pass or fail was good with κ=0.79 (0.53-1.00) and 90% accuracy. The two discrepant nets (net 5 and net 12) passed at IHI on KD60 but not M24 (Fig. 6). No nets with M24 exceeding 80% failed at either facility, while the majority of nets that passed at IHI, passed only on KD60 (Fig. 7).

Table 3: Contingency analysis for cone bioassays conducted in IHI and PNGIMR

Testing facilities		**PNGIMR**		
		Pass N (%)	**Fail N (%)**	**Total (%)**
IHI	**Pass**	12[a] (100)	2[c] (25)	14 (70)
	Fail	0[b](0)	6[d] (75)	6 (30)
	Total	12 (60)	8 (40)	20 (100)

'[a]' and '[d]' the number of nets agreed results at both testing facilities, '[b]' and '[c]' the number of nets with discrepant results between testing facilities

As indicated in Table 3, classify the n=20 ITNs (mean value of 5 net pieces from each ITN) into 'pass' and 'fail' categories based on mean values for M24 and KD60, using the WHO bioefficacy criteria and the inherent lower CI of the per-protocol cone bioassay as threshold for pass or fail.

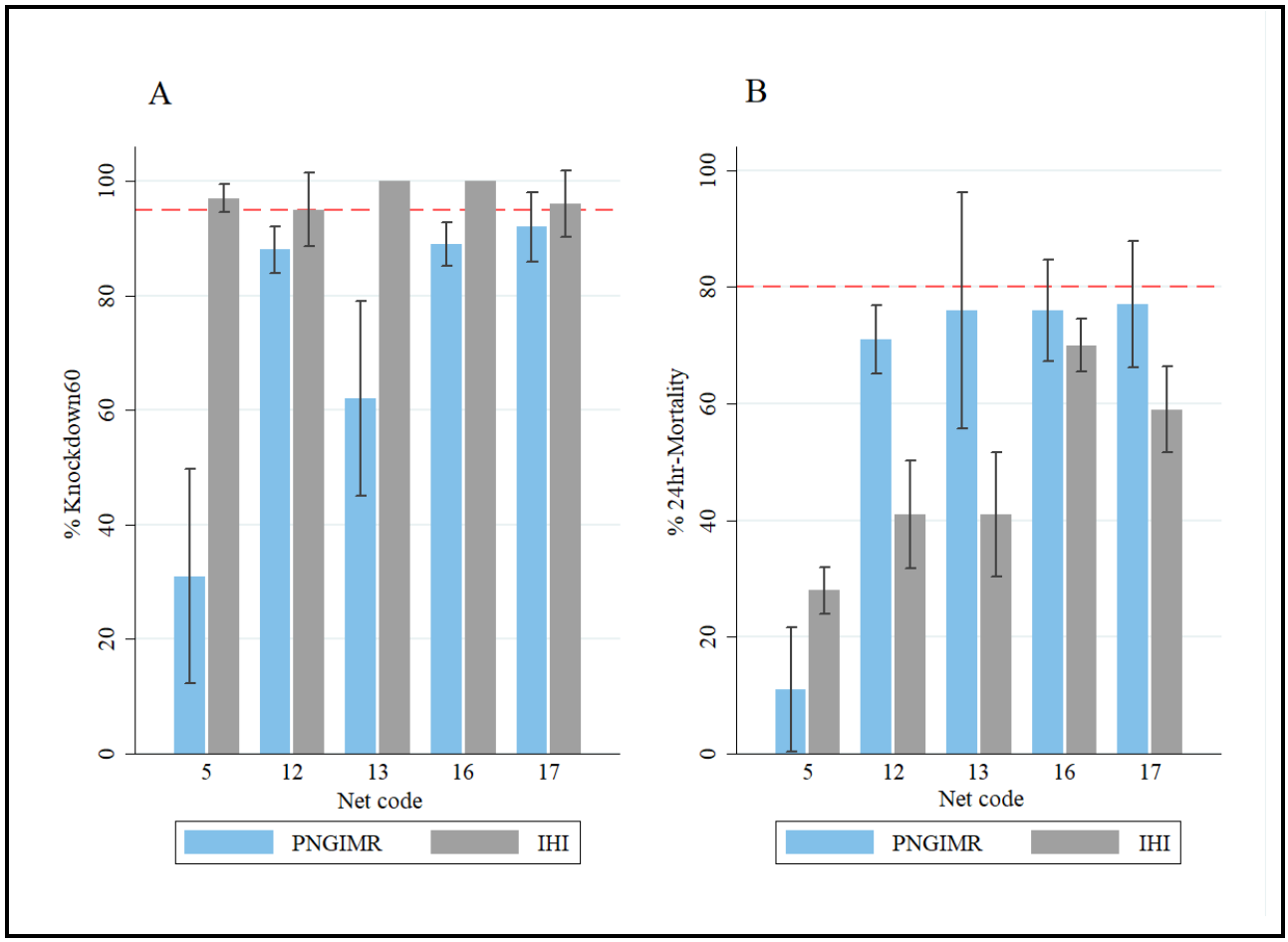

Figure 6: Bioefficacy of the five ITNs that demonstrated discordant results

As indicated in Fig. 6, each ITN passed efficacy criteria at IHI using the bioefficacy criterion of 95% KD60 (A) but did not reach the optimal bioefficacy criterion of 80% M24 (B). Three of the nets showed mean 24 hr mortality close to 80% at PNGIMR with confidence intervals that overlapped the optimal bioefficacy threshold of 80% mortality (B). Dashed lines are the WHO thresholds for 95% KD60 and 80% M24.

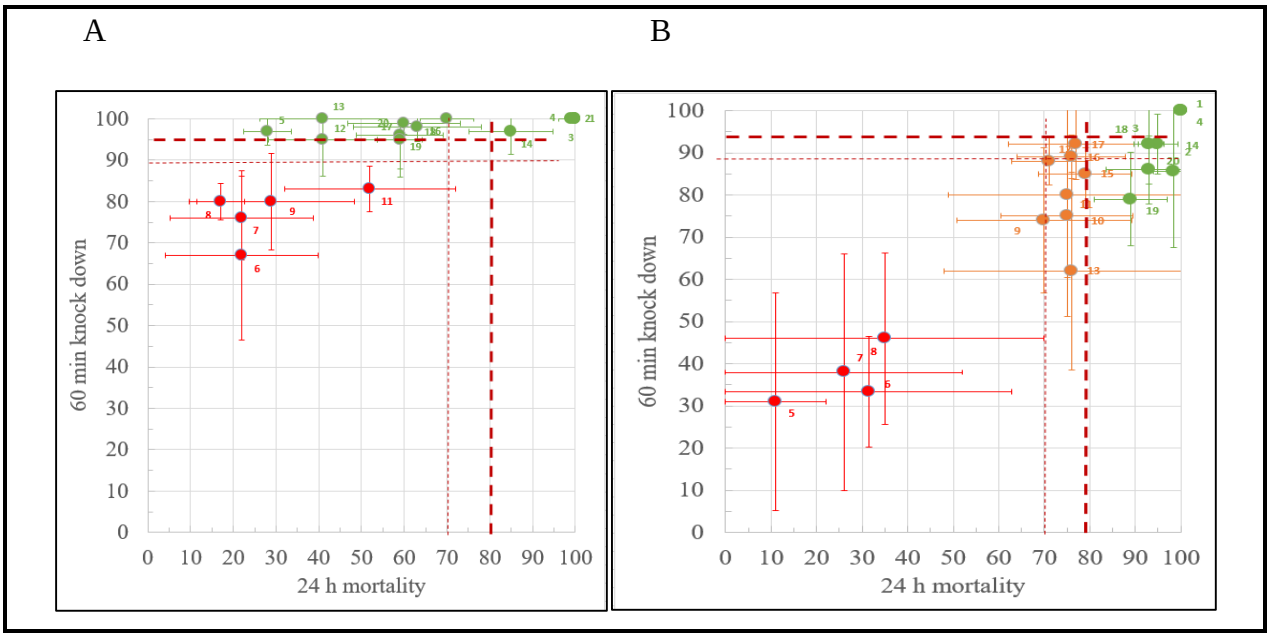

Figure 7: Correlation between M24 and KD60 at IHI (A) and PNGIMR (B)

As indicated in Fig. 7, ITNs passing (green) or failing (red) based on stringent cut-off WHO cone bioassay criteria of 80% M24 and 95% KD60. 'Borderline' nets for which the mean KD or M24 values are within the margin of stochastic error (95% CI) inherent to WHO cone bioassays based on the total number of mosquitoes used (n=100) are shown in amber. Thick dashed lines are the WHO thresholds 95% KD60 and 80% M24. Thin dashed lines indicate these assay-inherent 95% (lower) CIs of these thresholds.

4.6 Discussion

The present study explored the utility of cone bioassays for pre-delivery QA of pyrethroid ITNs in two test facilities using different *Anopheles* mosquitoes to test the assumption that cone bioassays are consistent and reproducible across locations, mosquito strains, and laboratories, and could be conducted in addition to physiochemical tests currently recommended for QA of ITNs. This study specifically compared the test results for pyrethroid ITNs from PNG using susceptible *An. gambiae s.s* and susceptible *An. farauti s.s.* WHO tunnel tests were used as a supplementary test in IHI to confirm bioefficacy of the nets that did not meet bioefficacy thresholds in cone bioassays. Consistency of bioefficacy results on tested ITNs between IHI and PNGIMR was demonstrated

(κ=79 and 90% accuracy), based on pass/fail categories, although absolute agreement between IHI and PNGIMR testing facilities was not observed, especially for those nets with low M24.

In this study, after modifying the pass criteria to account for inherent stochastic variation and systematic bias there was good agreement between the facilities indicating that the cone bioassay is a sensitive method to identify those nets with sufficient insecticide doses on the net surface to kill and incapacitate pyrethroid susceptible mosquitoes. It may, therefore, provide a means to identify nets with suboptimal insecticide doses on the net surface even using different *Anopheles* strains in different laboratories. Most previous studies identified from the literature review using cone bioassay tests reported bioefficacy above WHO critical thresholds for prior to use pyrethroid ITNs (Abílio *et al.*, 2015; Bhatt *et al.*, 2012; Castellanos *et al.*, 2021; Graham *et al.*, 2005; Kayedi *et al.*, 2007; Ketoh *et al.*, 2018; Malima *et al.*, 2013; Okia *et al.*, 2013; Rafinejad *et al.*, 2008; Tungu *et al.*, 2021; WHO, 2009a, 2015). However, a handful of studies reported bioefficacy below WHO critical thresholds in Benin (Ahogni *et al.*, 2019), Iran (Bagheri *et al.*, 2017), Madagascar (Randriamaherijaona *et al.*, 2017), and PNG (Vinit *et al.*, 2020). The reasons for this are unclear but our study corroborates the recent findings from PNG (Vinit *et al.*, 2020). However, it is known that cone bioassay results can be affected by ITN characteristics i.e. manufacturing processes and possibly poor shipping or storage conditions (AMP, 2020); as well as bioassay methods including sample preparation e.g. using a net sample straight from the fridge, mosquito age (Kulma *et al.*, 2013; Marti-Soler *et al.*, 2021) and fitness (Owusu *et al.*, 2017), test procedures (Owusu & Müller, 2016), temperature (Glunt *et al.*, 2018; Glunt *et al.*, 2014; Hodjati & Curtis, 1999a) and inter-operator variability (WHO, 2008).

As the cone test uses biological systems there are many factors that can affect the results obtained that need to be carefully controlled. These can be grouped into (a) mosquito rearing (b) infection control (c) environmental conditions (d) mosquito related factors and (e) conduct of the cone test.

Mosquito rearing: It is critical to standardise temperature because larval rearing temperature affects mosquito fitness and may alter their resistance to insecticides (Agyekum *et al.*, 2021). Rearing mosquitoes with an incorrect light dark cycle may decrease mosquito survival (Ukubuiwe *et al.*, 2018). Mosquito larval nutrition affects the size of mosquitoes and, therefore, may also affect

their susceptibility to insecticides (Kulma *et al.*, 2013). Optimal mosquito rearing procedures are outlined in the MR4 Guidelines (MR4, 2016).

Infection control: Preparation of the testing room and mosquito holding area before the conduct of the cone test is important. The laboratory and holding rooms need to be kept clean in order to prevent mosquito infection with microorganisms that may alter the observed mortality (Farenhorst *et al.*, 2009). Mosquito infection with pathogens reduces their host seeking and egg laying (Barnard *et al.*, 2007).

Environmental conditions: There is some evidence that humidity can also affect mosquito mortality observed after insecticide exposure (Kristan *et al.*, 2018) and it is known to affect mosquito survival (Schmidt *et al.*, 2018) and should therefore be carefully maintained during mosquito holding post-exposure. Mosquito detoxification has a periodicity (Balmert *et al.*, 2014) that follows the natural circadian rhythm of the mosquito (Yang *et al.*, 2010) so it is important to conduct studies at a similar time each day to minimize heterogeneity between observations.

Mosquitoes: Using mosquitoes with standardised age and nutritional status is essential to allow the comparability of assays between laboratories. The age (Hodjati & Curtis, 1999b; Machani *et al.*, 2019), nutritional status (Machani *et al.*, 2019) and sugar (Norris & Bloomquist, 2021) of mosquitoes alters their susceptibility to insecticides. The time that a mosquito received a blood meal relative to contact with an insect growth regulator or juvenile hormone analogue can impact the results of the bioassay (Grisales *et al.*, 2021). Careful transport of mosquitoes from the insectary to the test room in sealed containers and allowing mosquitoes to acclimate to the test room before bioassay will minimize physiological stress and its effects on metabolic and physiological status and so avoid possible bias in observed mortality. It is important to avoid overuse of the colony so that the colony becomes depleted and individual mosquito fitness is compromised.

Conduct of cone test: For ITN samples that are refrigerated, allowing the ITNs to return to room temperature before testing is important. This is because pyrethroids have a temperature dependent toxicity (Khambay & Jewess, 2005) and failure to test the ITNs at the correct temperature may introduce bias into the observed mortality. The angle at which the WHO cone bioassay is

performed considerably affects the time mosquitoes spend in contact with the net, and subsequently 24 h mortality. It is advised to use the cone test at 45º or 60º angle to maximize mosquito contact with the treated surface of the ITN (Owusu & Müller, 2016). Placement of ITN samples on the board should be done without stretching or bunching the ITN material as this affects the amount of treated netting under the cone and consequently the treated surface is available to the mosquito. To enable comparability of results from different test facilities, standard cone (12 cm diameter, available from WHO) should be used to standardize the surface area of netting available to mosquitoes. Cutting a hole in the board and using plastic stoppers so that mosquitoes can only rest on the ITN sample for the exposure time (as done at IHI) helps to minimize heterogeneity in results. For the purposes of comparability between testing facilities and time points, it is critical to evaluate ITNs at a standard temperature of 27±2ºC. Conducting studies at a different temperature can affect the observed results. A bimodal temperature-activity distribution has been reported in several insecticides and mosquito species (Beach *et al.*, 1989; Glunt *et al.*, 2018; Hodjati & Curtis, 1999a; Whiten & Peterson, 2016) and 27±2ºC gives a conservative measurement of mortality. Temperature affects the way in which pyrethroids work in insects. Initial symptoms of Type I pyrethroids are positively correlated with temperature, the toxic action (release of neurotransmitter and conduction block) is negatively correlated with temperature (Khambay & Jewess, 2005) whereas other insecticide classes tend to become more toxic at higher temperatures (Oxborough *et al.*, 2015).

Discrepant results obtained for the absolute KD60 or M24 values measured between facilities for the same ITN sample is likely to be due to random errors and/or systematic bias in studies. Similar differences have been observed in other multi-centre studies to compare three test methods in determining the bioefficacy of the same nets (WHO, 2007). Some of the observed differences are likely to be due to testing conditions, procedures, and the different mosquito strains at the two facilities. Differences that cannot be ruled out are temperature which is known to impact mortality (Glunt *et al.*, 2018; Glunt *et al.*, 2014). The temperature in PNG was 28±4°C compared to 27±1°C at IHI, although control mortality was acceptable at both sites. Variability in cone bioassay procedures i.e. the angle of cone was 45° (Vinit *et al.*, 2020) as per WHO guidelines in PNGIMR, while in IHI the cone test is performed at 60° in the cone assay board to maximize mosquito contact with ITNs although this has been shown to be inconsequential (Owusu & Müller, 2016). Net pieces

were shipped to Tanzania from PNG by courier in an insulated package with a very short transit time. As such it is unlikely that transport would have affected their bioefficacy. Operator skill may have contributed to the variability of results, but it should be noted that cone bioassays conducted on the same pieces at different time points gave similar results. The *An. gambiae s.s* and *An. farauti s.s* strains used were fully susceptible to pyrethroid insecticides, of a similar age, and exposed to similar colony maintenance conditions; these strains are not sibling species and they have differing morphology (Manguin, 2013). The *An. gambiae* strain used for this test has shown high mortality in WHO cone bioassays against several ITN brands including the ones tested in this current study. Mosquito strain variability e.g., size and fitness may explain some of the variation in absolute values measured. Both strains were assessed for insecticide resistance at the time of testing. Both strains were fully susceptible to pyrethroids at 1x WHO diagnostic concentration (WHO, 2016), but it is likely that the concentrations needed to knock down but not kill *An. gambiae s.s.* (Ifakara) are lower than the 1x diagnostic concentration. It is currently unclear how the choice of susceptible laboratory-reared mosquito strains affects the outcomes of WHO cone bioassays and more research is needed to establish robust parameters for comparison. Even so, the WHO cone bioassays in the present study showed very good agreement for nets that demonstrated the highest M24. Unsurprisingly, more variation in results was observed between the testing facilities for ITNs with low KD60 or M24. This is a well-known phenomenon and for this reason, large sample sizes (30-50 nets) are recommended for cone testing used for bioefficacy monitoring of field used ITNs that generally have reduced M24 (WHO, 2013a).

In the present study, five of the 20 prior to use ITNs effectively killed mosquitoes (≥80% M24) at IHI. The average M24 measured in cone bioassay (mean from the two sites) for the best and the worst performing prior to use ITNs was 99% and 24%, respectively. These results agree well with other studies and WHO specification reports (Ahogni *et al.*, 2019; Bagheri *et al.*, 2017; Randriamaherijaona *et al.*, 2017; Vinit *et al.*, 2020; WHO, 2007, 2009a). Even so, most ITNs tested at IHI gave higher KD60 than M24. It has been observed that with *An. gambiae* to achieve 80% M24 requires at least a 5% higher net surface concentration of pyrethroid than to achieve 95% KD60 (WHO, 2008). In a WHOPES report (2008) it was found that for *An. gambiae* the KD60 criterion is met at dosages lower than the M24 criterion, so that 95% KD60 corresponds to 20-30% M24 (WHO, 2008). It was reported in an expert review that new prior to use deltamethrin

coated nets demonstrate 100% KD60 but 55% M24 (WHO, 2009a). It may, therefore, be inferred that M24 is the more conservative endpoint of pyrethroid performance in a cone bioassay. Indeed, it was previously stipulated by WHOPES that as the two existing WHO criteria for biological effect in the cone test correspond to different surface concentrations of the active ingredient, they are not equivalent, and one of them should be designated as the basis for WHO specifications. Possibly the criterion could be chosen on a case-by-case basis but mortality is clearly more stringent than KD and therefore appears to be the criterion of choice (WHO, 2008).

The data from this study corroborate this, and mortality was the more stringent criterium in this work. All analyses conducted in the present study showed greater agreement between the two sites when M24 was used as the endpoint. Spearman correlation showed a very strong correlation of efficacy results for M24 (r=0.9) between the two testing facilities and the Bland Altman showed more consistent agreement on this endpoint. These results further corroborate other confirmatory analyses of PNGIMR bioefficacy tests conducted at Liverpool School of Tropical Medicine (LSTM) where susceptible *An. gambiae s.s.* mortality estimates were strongly correlated with PNGIMR results (coefficient of determination equal to 0.80) (Vinit *et al.*, 2020). Five of 20 (25%) of nets had discrepant results between IHI and PNGIMR testing facilities. The number of discrepant results is further decreased if assay-inherent stochastic variability is considered. It should be noted that analysis in this study observed differences in ITN bioefficacy when considering individual net pieces. Because each net piece has only 4 cones there is even greater heterogeneity for comparisons of net pieces. Due to the lower number of replicates the 95% CI of the proportion for the 80% M24 is 58%-93% and for KD60 75% - 100%. It is therefore necessary to consider comparison of the combined pieces for each ITN that have a total of 20 replicates each to give a more precise estimation of bioefficacy. There are variations in spatial presentation and/or distribution of active ingredients within the netting, or the surface treatment. This is well recognised as the WHOPES report states a consequence of the narrow dose ranges over which biological responses change dramatically is that responses cut-off values for decision-making are inevitably set within a region in which small errors in measurements can have a disproportionately large impact. This problem is compounded by the high sampling error associated with the very variable active ingredient distribution in many types of insecticidal netting (WHO, 2008). The current work corroborates this statement and for this reason the use of confidence intervals that

reflect the natural variability in the bioassay based on the number of replicates used for evaluation is a useful addition to thresholds for bioefficacy criteria. Furthermore, the assay inherent uncertainty should be better accounted for. In this study we used a simple method based on estimates of the 95% CIs around the WHO thresholds of M24 and KD60 when 20 cones i.e., n=100 mosquitoes are used. Nets with WHO cone bioassay results that fell within this margin of assay-inherent error were still considered as passed. However, precision of the pass/fail could also be improved by increasing sample size and this study suggests that larger sample sizes for QA testing are appropriate.

In this study, however, all nets passed tunnel tests, possibly because of longer mosquitoes exposure time (12 hours) compared to the cone assay test (3 minutes) as well as sugar starvation in the tunnel test (WHO, 2013a). Given that it provides the least stringent evaluation and requires the most complex setup, the need for tunnel tests for testing pyrethroid ITN bioefficacy is questionable and may be a means for ITNs with lower surface concentrations of deltamethrin insecticide to pass WHO bioefficacy criteria (Hougard *et al.*, 2003).

In the present study, four pyrethroid ITN brands were included. All of these brands had passed WHOPES testing and were recommended (now pre-qualified) based on WHO cone bioassay data. Several brands were selected to increase the generalizability of the findings. Our systematic review highlighted that all these brands had passed bioefficacy criteria in the WHO cone bioassays in multiple studies. The results from this study agreed with the results of several studies of PermaNet®2012 (Castellanos *et al.*, 2021; Graham *et al.*, 2005; Kilian *et al.*, 2008; Kweka *et al.*, 2017; Norris & Norris, 2011), and PermaNet®2019 nets (Bagheri *et al.*, 2017; Vinit *et al.*, 2020). The Yorkool® nets results are similar to WHO prequalification reports (WHO, 2007, 2009a) and recent results from durability studies in Benin (Ahogni *et al.*, 2019) and Madagascar (Randriamaherijaona *et al.*, 2017). For the Interceptor® and SafeNet® nets, cone bioassay results in this study were lower than that seen in other studies (Camara *et al.*, 2018; Malima *et al.*, 2013; Tungu *et al.*, 2021; WHO, 2007, 2015). Some variability in the surface bioavailability of pyrethroids on ITN samples may be introduced by the manufacturing process (Graham *et al.*, 2005; Kilian *et al.*, 2008; Müller *et al.*, 2002), variations in spatial presentation and/or distribution of active ingredient within the netting. Net surface bioavailability of pyrethroids can also be affected

by insecticide migration rate (WHO, 2008), poor storage or shipping conditions and the binder used (Vinit *et al.*, 2020). However, we consider it a minimum standard for ITNs to have adequate surface concentration to kill pyrethroid susceptible mosquitoes when they are new.

In this study, the systematic review showed that in some countries with a high malaria burden, e.g. Nigeria, ITN QA using a WHO cone bioassays was introduced after a long period of importation of nets with low bioefficacy (Daniel, 2006). It is important for ITN bioefficacy to be evaluated post shipment to ensure that nets procure will perform as required. Acceptable performance of ITNs is defined by WHO as retention of biological activity (e.g. M24 ≥80%) through 20 standard washes (or 3 years of use) but there is no simple physiochemical measurement corresponding to this definition (WHO, 2008). It is generally agreed that a validated, low-cost, easy-to-implement laboratory methodology for assessing surface AI content is urgently needed (LLP, 2021) but current methods have not been found to correspond well to bioefficacy results (Villalta *et al.*, 2021). World Health Organization cone bioassays have been demonstrated to be highly sensitive to changes of active ingredient concentration on the net surface and could thus play a crucial role in ITN QA (WHO, 2007). However, many endemic countries do not have well-established cone bioassays for ITN QA (either as post-delivery or pre-distribution QA). Cone bioassays were recommended for QA of conventionally treated nets (Jawara *et al.*, 1998; Lengeler *et al.*, 1996). The tenth WHOPES meeting report of 2006 following the interlaboratory evaluation of cone bioassays tested in 5 different laboratories on same nets organized by WHO recommended a standard cone bioassay to be used for ITN QA purposes until an alternative was developed (WHO, 2007). Although, in this interlaboratory evaluation report included the same nets but provided a different bioefficacy results observed on deltamethrin unwashed nets and washed nets may pass from 3 to 6 washes, which is a difference of 100% (these were dipped nets made by one of the laboratories and sent to all, not LLIN) (WHO, 2007). The eleventh WHOPES meeting, however, concluded that WHO standard bioassays cannot be used throughout the world for ITN QA purposes, so physicochemical tests must be used instead following reasons that WHO cone bioassay outcomes showed variation and were mosquito strain dependent (WHO, 2008). The current work adds weight to the argument that the choice of the mosquito strain or differences between laboratories systematically affects the WHO cone bioassays results. We show that WHO cone bioassays are reproducible if the systematic bias is accounted for. This can easily be achieved

by conducting studies such as this one but requires partnership between testing facilities and flexibility from policymakers. Further harmonisation of laboratory methods may also assist in minimising inter-facility differences in results. More evidence is needed to test whether M24 criteria should be mosquito species specific (although it should be noted that some nets achieved >80% mortality with both strains). This can be likened to the already existing species-specific guidance on discriminatory insecticide concentrations used in WHO tube bioassays (WHO, 2016). Therefore, well-controlled bioassays can be used for QA purposes if there is a will to address the complex realities.

The recent landscape bioefficacy report (LLP, 2021) and several other studies (Calle *et al.*, 2018; Karl *et al.*, 2021; Lindsay *et al.*, 2021; Wheldrake *et al.*, 2021) have highlighted the need for better QA. Almost all the studies found in the systematic review showed high KD60 and M24 of prior to use pyrethroid ITNs with pyrethroid susceptible strains. While it could be that there is a bias toward the publication of positive trials, the inclusion of the WHOPES reports, and several independent operational monitoring studies suggests that this is not the case. A few independent operational monitoring reports revealed that ITNs that did not pass bioefficacy thresholds were distributed to the endemic population however, these nets had passed the prequalification process with demonstrated high bioefficacy.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Based on these study findings, the WHO cone bioassay is a reproducible bioassay provided inherent stochastic variation and systematic bias are accounted for and agree well where nets pass WHO M24 thresholds. The systematic review included in this study confirms that WHO cone bioassay bioefficacy criteria have been previously achieved by all pyrethroid ITNs (unwashed), without the need for additional tunnel tests. The 80% M24 threshold remains the most reliable indicator of pyrethroid ITN quality among pyrethroid susceptible mosquitoes.

5.2 Recommendations

From the conclusion, the following recommendations were made:

(i) The National Malaria Control Program (NMCP) and other agencies in malaria endemic countries to strengthen quality assurance of vector control tools and incorporate cone tests for ITNs bioefficacy check on pre-delivery inspections and/or prior to distribution.

(ii) It is critical that WHO resumes reporting ITN performance data in prequalification reports to be used as a product performance reference by procurement agencies, the NMCP or other bodies that monitor product performance at a country level.

(iii) Also of note, many publications and reports did not indicate country of manufacture, ITN age, and lot/batch of the tested nets or data collection period. This information is useful to aid procurement agencies or manufacturers to investigate any possible product failures and pinpoint probable causes such as poor shipping, storage or batch variability.

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APPENDICES

Table 4: Summary of data from systematic review on cone bioassays

Author/Report	Location	Pyrethroid ITN	Active Ingredients	Production technology	Year	Susceptible mosquito strains	KD60	M24
Abilio and colleagues, 2015 (Abílio *et al.*, 2015)	Mozambique	Interceptor ®	Alpha-cypermethrin	Impregnation		*An. arabiensis*	80.56	98.84
Abilio and colleagues, 2015 (Abílio *et al.*, 2015)	Mozambique	PermaNet ® 2.0	Deltamethrin	Impregnation		*An. arabiensis*	94.72	100
Abilio and colleagues, 2015 (Abílio *et al.*, 2015)	Mozambique	Olyset Net®	Permethrin	Incorporation		*An. arabiensis*	68.33	90.36
Agossa and colleagues, 2014 (Agossa *et al.*, 2014)	Benin	PermaNet ® 2.0	Deltamethrin	Impregnation		*An. gambiae* (Kisumu strain)	100	100
Ahogni and colleagues, 2019 (Ahogni *et al.*, 2019)	Benin	Yorkool®	Deltamethrin	Impregnation	2017	*An. gambiae* (Kisumu strain)	62	74
Allossogbe and colleagues, 2017 (Allossogbe *et al.*, 2017)	Benin	PermaNet ® 2.0	Deltamethrin	Impregnation	2015-2016	*An. gambiae* (Kisumu strain)	93.33	100
Allossogbe and colleagues, 2017 (Allossogbe *et al.*, 2017)	Benin	Olyset Net®	Permethrin	Incorporation	2015-2016	*An. gambiae* (Kisumu strain)	100	100

Bagheri and colleagues, 2017(Bagheri *et al.*, 2017)	Iran	PermaNet ® 2.0	Deltamethrin	Impregnation	2016	*An. stephensi*	74	22
Bhatt and colleagues, 2012 (Bhatt *et al.*, 2012)	India	Interceptor ®	Alpha-cypermethrin	Impregnation	2006	*An. culicifacies*	96.7	100
Camara and colleagues, 2018 (Camara *et al.*, 2018)	Côte d'Ivoire	Interceptor ®	Alpha-cypermethrin	Impregnation		*An. gambiae* (Kisumu strain)	97	99
Castellanos and colleagues, 2021 (Castellanos *et al.*, 2021)	Guatemala	PermaNet ® 2.0	Deltamethrin	Impregnation	2012	*An. albimanus*	100	100
Clegban and colleagues, 2021 (Clegban *et al.*, 2021)	Côte d'Ivoire	Yahe®	Deltamethrin	Impregnation	2014	*An. gambiae* (Kisumu strain)	100	97.2
Clegban and colleagues, 2021 (Clegban *et al.*, 2021)	Côte d'Ivoire	PandaNet ® 2.0	Deltamethrin	Incorporation	2014	*An. gambiae* (Kisumu strain)	100	100
Graham and colleagues, 2005 (Graham *et al.*, 2005)	Iran	PermaNet ® 2.0	Deltamethrin	Impregnation	2000	*An. stephensi* (Beech strain)	100	97.7
Kilian and colleagues, 2008 (Kilian *et al.*, 2008)	Montpellier, France	PermaNet ® 1.0	Deltamethrin	Impregnation	2000	*An. gambiae* (Kisumu strain)	95	80

Kilian and colleagues, 2008 (Kilian *et al.*, 2008)	CDC Atlanta, USA	PermaNet ® 2.0	Deltamethrin	Impregnation	2002	*An. gambiae* (Kisumu strain)	95	80
Kweka and colleagues, 2011 (Kweka *et al.*, 2011)	Tanzania	PermaNet ® 2.0	Deltamethrin	Impregnation	2005	*An. gambiae* (Kisumu strain)	100	100
Kweka and colleagues, 2017 (Kweka *et al.*, 2017)	Tanzania	PermaNet ® 2,0	Deltamethrin	Impregnation		*An. gambiae* (Kisumu strain)	100	100
Kweka and colleagues, 2019 (Kweka *et al.*, 2019)	Tanzania	MagNet®	Alpha-cypermethrin	Incorporation		*An. gambiae* (Kisumu strain)	90.4	100
Kweka and colleagues, 2019 (Kweka *et al.*, 2019)	Tanzania	DuraNet®	Alpha-cypermethrin	Incorporation		*An. gambiae* (Kisumu strain)	100	100
Mahande and colleagues, 2018 (Mahande *et al.*, 2018)	Tanzania	DuraNet®	Alpha-cypermethrin	Incorporation	2015	*An. gambiae* (Kisumu strain)	100	100
Malima and colleagues, 2013 (Malima *et al.*, 2013)	Tanzania	Interceptor ®	Alpha-cypermethrin	Impregnation		*An. gambiae s.l*	100	100
Mussa and colleagues, 2020 (Jeremiah J Musa *et al.*, 2020)	Tanzania	DawaPlus ®	Deltamethrin	Impregnation	2019	*An. gambiae*	100	92.5
Ngufor and colleagues, 2020 (Ngufor *et al.*, 2020)	Benin	Royal Sentry®	Alpha-cypermethrin	Incorporation		*An. gambiae* (Kisumu strain)	100	98

Okia and colleagues, 2013 (Okia *et al.*, 2013)	Uganda	PermaNet ® 2.0	Deltamethrin	Impregnation	Started 2011	*An. gambiae* (Kisumu strain)	100	100
Okia and colleagues, 2013 (Okia *et al.*, 2013)	Uganda	Interceptor ®	Alpha-cypermethrin	Impregnation	Started 2011	*An. gambiae* (Kisumu strain)	95	100
Okia and colleagues, 2013 (Okia *et al.*, 2013)	Uganda	Olyset Net®	Permethrin	Incorporation	started 2011	*An. gambiae* (Kisumu strain)	100	100
Pennetier and colleagues, 2013 (Pennetier *et al.*, 2013)	Malanville, Benin	Olyset Net®	Permethrin	Incorporation		*An. gambiae* (Kisumu strain)	64	100
Rafinejad and colleagues, 2008 (Rafinejad *et al.*, 2008)	Iran	PermaNet ®	Deltamethrin	Impregnation		*An. stephensi*	100	94.9
Rafinejad and colleagues, 2008 (Rafinejad *et al.*, 2008)	Iran	Olyset Net®	Permethrin	Incorporation		*An. stephensi*	100	97
Randriamaherijaona and colleagues, 2017 (Randriamaherijaona *et al.*, 2017)	Madagascar	Royal Sentry®	Alpha-cypermethrin	Incorporation		*An. arabiensis*	100	90.2
Sood and colleagues, 2011 (Sood *et al.*, 2011)	India	PermaNet ® 2.0	Deltamethrin	Impregnation		*An. stephensi*	100	100

Sood and colleagues, 2011 (Sood *et al.*, 2011)	India	Olyset Net®	Permethrin	Incorporation		*An. stephensi*	100	100
Vinit and colleagues, 2020 (Vinit *et al.*, 2020)	Papua New Guinea	PermaNet ® 2.0	Deltamethrin	Impregnation	Between 2007 and 2012	*An. farauti*	96.48	98.72
Vinit and colleagues, 2020 (Vinit *et al.*, 2020)	Papua New Guinea	PermaNet ® 2.0	Deltamethrin	Impregnation	Between 2013 and 2019	*An. farauti*	41.23	40.12
WHO, 2004 (WHO, 2004b)	Montipellier, France	PermaNet ® 1.0	Deltamethrin	Impregnation	2-4 December, 2003	*An. gambiae* (Kisumu strain)	100	100
WHO, 2004 (WHO, 2004b)	Benin	PermaNet ® 2.0	Deltamethrin	Impregnation	2-4 December, 2003	*An. gambiae* (Kisumu strain)	100	100
WHO, 2004 (WHO, 2004b)	Montipellier, France	PermaNet ® 1.0	Deltamethrin	Impregnation	2-4 December, 2003	*Cx. quinquefasciatus*	100	100
WHO, 2007 (WHO, 2007)	Malanville, Benin	Interceptor ®	Alpha-cypermethrin	Impregnation	11-14 December, 2006	*An. gambiae*	100	100
WHO, 2007 (WHO, 2007)	Montipellier, France	Hiking Group®	Deltamethrin	Impregnation	11-14 December, 2006	*An. gambiae* (Kisumu)	100	95
WHO, 2007 (WHO, 2007)	Montipellier, France	Yorkool®	Deltamethrin	Impregnation	11-14 December, 2006	*An. gambiae* (Kisumu)	83	16
WHO, 2007 (WHO, 2007)	Montipellier, France	Netto Group®	Deltamethrin	Impregnation	11-14 December, 2006	*An. gambiae* (Kisumu)	95	100
WHO, 2007 (WHO, 2007)	Montipellier, France	PermaNet ® 2.0	Deltamethrin	Impregnation	11-14 December, 2006	*An. gambiae* (Kisumu)	100	100

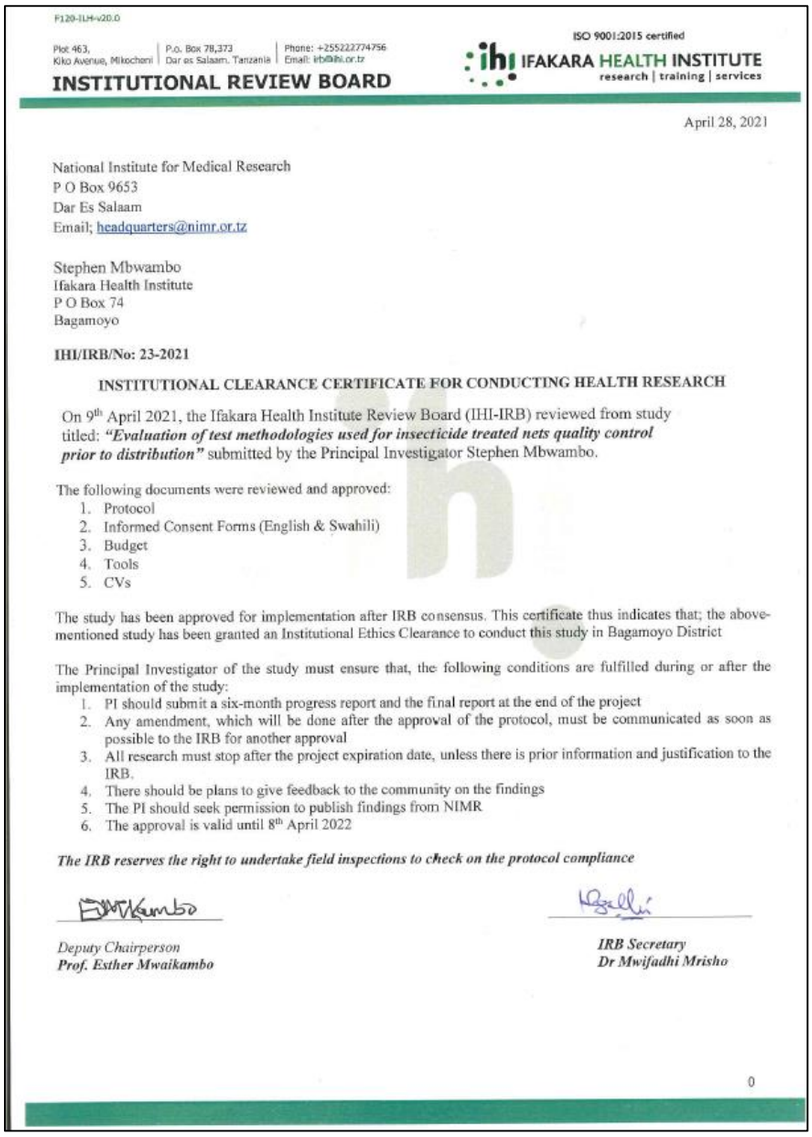
WHO, 2008 (WHO, 2008)	Kyenjonjo, Uganda	PermaNet ® 2.0	Deltamethrin	Impregnation	11-14 December, 2006	*An. gambiae* (Kisumu)	95	95
WHO, 2008 (WHO, 2008)	Montipellier, France	Dawaplus ®	Deltamethrin	Impregnation	10-13 December, 2007	*An. gambiae* (Kisumu)	93	39
WHO, 2008 (WHO, 2008)	Kou Valley, Bukina Faso	Netprotect ®	Deltamethrin	Incorporation	10-13 December, 2007	*An. gambiae* (Kisumu)	100	100
WHO, 2008 (WHO, 2008)	Kou Valley, Bukina Faso	DuraNet®	Alpha-cypermethrin	Incorporation	10-13 December, 2007	*An. gambiae* (Kisumu)	100	100
WHO, 2008 (WHO, 2008)	WHOPES supervised studies	DuraNet®	Alpha-cypermethrin	Incorporation	10-13 December, 2007	*An. gambiae* (Kisumu)	100	98
WHO, 2008 (WHO, 2008)	Muheza, Tanzania	DuraNet®	Alpha-cypermethrin	Incorporation	10-13 December, 2007	*An. gambiae* (Kisumu)	100	100
WHO, 2009 (WHO, 2009b)	Melanville, North Benin	PermaNet ® 2.5	Deltamethrin	Impregnation	8-11 December, 2008	*An. gambiae* (Kisumu)	100	100
WHO, 2009 (WHO, 2009b)	Melanville, North Benin	PermaNet ® 2.0	Deltamethrin	Impregnation	8-11 December, 2008	*An. gambiae* (Kisumu)	100	100
WHO, 2009 (WHO, 2009b)	Kilimanjaro district, Tanzania	PermaNet ® 2.5	Deltamethrin	Impregnation	8-11 December, 2008	*An. gambiae* (Kisumu)	100	100
WHO, 2009 (WHO, 2009b)	Kilimanjaro district, Tanzania	PermaNet ® 2.0	Deltamethrin	Impregnation	8-11 December, 2008	*An. gambiae* (Kisumu)	100	100
WHO, 2010 (WHO, 2009a)	Montipellier, France	Yorkool®	Deltamethrin	Impregnation	28–30 July, 2009	*An. gambiae* (Kisumu)	100	55

WHO, 2010 (WHO, 2009a)	Montipellier, France	PermaNet ® 2.0	Deltamethrin	Impregnation	28–30 July, 2009	*An. gambiae* (Kisumu)	100	100
WHO, 2010 (WHO, 2009a)	Malanville, Benin	DawaPlus ®	Deltamethrin	Impregnation	28–30 July, 2009	*An. gambiae* (Kisumu)	100	100
WHO, 2010 (WHO, 2009a)	Malanville, Benin	DawaPlus ® 2.0	Deltamethrin	Impregnation	28–30 July, 2009	*An. gambiae* (Kisumu)	100	100
WHO, 2010 (WHO, 2009a)	Muheza, Tanzania	DawaPlus ® 2.0	Deltamethrin	Impregnation	28–30 July, 2009	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)	Montipellier, France	Yahe®	Deltamethrin	Impregnation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)	Montipellier, France	PermaNet ® 2.0	Deltamethrin	Impregnation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)	Montipellier, France	Royal Sentry®	Alpha-cypermethrin	Incorporation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)	Montipellier, France	DuraNet®	Alpha-cypermethrin	Incorporation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)	Montipellier, France	MagNet®	Alpha-cypermethrin	Incorporation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2011 (WHO, 2011)		LifeNet®	Deltamethrin	Incorporation	11–15 April, 2011	*An. gambiae* (Kisumu)	100	100
WHO, 2012 (WHO, 2012)	India	Interceptor ®	Alpha-cypermethrin	Impregnation	18–22 June, 2012	*An. culicifacies*	97.8	98
WHO, 2012 (WHO, 2012)	Muheza, Tanzania	Interceptor ®	Alpha-cypermethrin	Impregnation	18–22 June, 2012	*An. gambiae* (Kisumu)	100	99

WHO, 2012 (WHO, 2012)	Benin	Olyset Net®	Permethrin	Incorporation	18–22 June, 2012	*An. gambiae* (Kisumu)	100	37
WHO, 2013 (WHO, 2013b)	Mae Sot District, Thailand	PermaNet ® 2.0	Deltamethrin	Impregnation	22–30 July, 2013	*An. culicifacies*	100	100
WHO, 2013 (WHO, 2013b)	Muheza, Tanzania	Yahe®	Deltamethrin	Impregnation	22–30 July, 2013	*An. gambiae* (Kisumu)	100	100
WHO, 2013 (WHO, 2013b)	Mae Sot District, Thailand	Yahe®	Deltamethrin	Impregnation	22–30 July, 2013	*An. minimus*	73	58
WHO, 2013 (WHO, 2013b)	Muheza, Tanzania	PermaNet ® 2.0	Deltamethrin	Impregnation	22–30 July, 2013	*An. gambiae* (Kisumu)	100	100
WHO, 2013 (WHO, 2013b)	Rourkela, India	DuraNet®	Alpha-cypermethrin	Incorporation	22–30 July, 2013	*An. culicifacies*	100	100
WHO, 2015 (WHO, 2015)	WHOPES supervised studies	Yahe®	Deltamethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	93	97
WHO, 2015 (WHO, 2015)	Montipellier, France	SafeNet®	Alpha-cypermethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	100	99.5
WHO, 2015 (WHO, 2015)	Montipellier, France	SafeNet®	Alpha-cypermethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	100	100
WHO, 2015 (WHO, 2015)	Montipellier, France	Interceptor ®	Alpha-cypermethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	100	100
WHO, 2015 (WHO, 2015)	Montipellier, France	PermaNet ® 2.0	Deltamethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	100	100

WHO, 2015 (WHO, 2015)	Côte d'Ivoire	PermaNet ® 2.0	Deltamethrin	Impregnation	29 June-1 July, 2015	*An. gambiae* (Kisumu)	100	100
WHO, 2015 (WHO, 2015)	WHOPES supervised studies	MiraNet®	Alpha-cypermethrin	Incorporation	29 June– 1 July, 2015	*An. gambiae* (Kisumu)	100	100
WHO, 2015 (WHO, 2015)	Côte d'Ivoire	Panda Net® 2.0	Deltamethrin	Incorporation	29 June– 1 July, 2015	*An. gambiae* (Kisumu)	100	100
WHO, 2015 (WHO, 2015)	WHOPES supervised studies	Panda Net® 2.0	Deltamethrin	Incorporation	29 June– 1 July, 2015	*An. gambiae* (Kisumu)	97	51
WHO, 2019 (WHO, 2019)	Ifakara Health Institute, Tanzania	Royal Sentry® 2.0	Alpha-cypermethrin	Incorporation	2017	*An. gambiae* (Ifakara strain)	100	100
WHO, 2020 (WHO, 2020)	Reference laboratory	Tsara Soft®	Deltamethrin	Incorporation	2019	*An. dirus*	95	80

(a) Institutional Clearance Certificate



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IFAKARA HEALTH INSTITUTE
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INSTITUTIONAL REVIEW BOARD

April 28, 2021

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Stephen Mbwambo
Ifakara Health Institute
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Bagamoyo

IHI/IRB/No: 23-2021

INSTITUTIONAL CLEARANCE CERTIFICATE FOR CONDUCTING HEALTH RESEARCH

On 9th April 2021, the Ifakara Health Institute Review Board (IHI-IRB) reviewed from study titled: ***"Evaluation of test methodologies used for insecticide treated nets quality control prior to distribution"*** submitted by the Principal Investigator Stephen Mbwambo.

The following documents were reviewed and approved:

1. Protocol
2. Informed Consent Forms (English & Swahili)
3. Budget
4. Tools
5. CVs

The study has been approved for implementation after IRB consensus. This certificate thus indicates that; the above-mentioned study has been granted an Institutional Ethics Clearance to conduct this study in Bagamoyo District

The Principal Investigator of the study must ensure that, the following conditions are fulfilled during or after the implementation of the study:

1. PI should submit a six-month progress report and the final report at the end of the project
2. Any amendment, which will be done after the approval of the protocol, must be communicated as soon as possible to the IRB for another approval
3. All research must stop after the project expiration date, unless there is prior information and justification to the IRB.
4. There should be plans to give feedback to the community on the findings
5. The PI should seek permission to publish findings from NIMR
6. The approval is valid until 8th April 2022

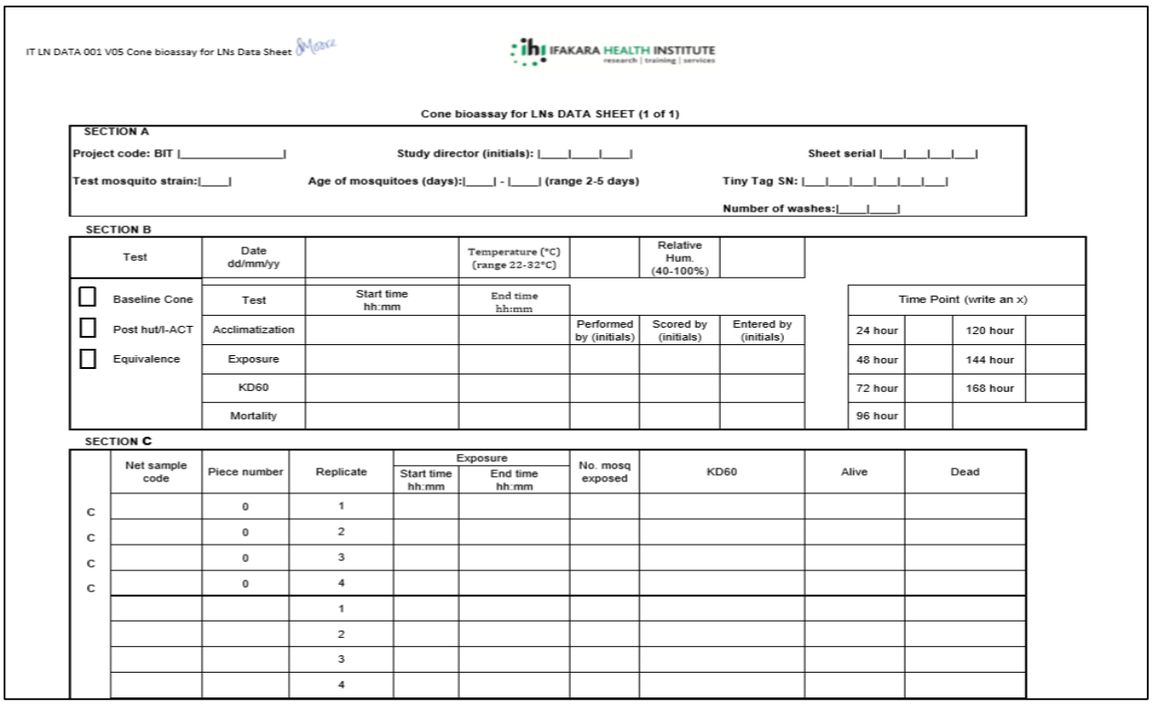
The IRB reserves the right to undertake field inspections to check on the protocol compliance

Deputy Chairperson
Prof. Esther Mwaikambo

IRB Secretary
Dr Mwifadhi Mrisho

0

(b) Data collection tools (WHO cone bioassays and tunnel tests)



IT LN DATA 001 V05 Cone bioassay for LNs Data Sheet

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Cone bioassay for LNs DATA SHEET (1 of 1)

SECTION A

Project code: BIT |_____| Study director (initials): |____|____|____| Sheet serial |___|___|___|___|

Test mosquito strain:|____| Age of mosquitoes (days):|____| - |____| (range 2-5 days) Tiny Tag SN: |___|___|___|___|___|___|

Number of washes:|____|____|

SECTION B

Test	Date dd/mm/yy		Temperature (°C) (range 22-32°C)		Relative Hum. (40-100%)	
☐ Baseline Cone	Test	Start time hh:mm	End time hh:mm			
☐ Post hut/I-ACT	Acclimatization			Performed by (initials)	Scored by (initials)	Entered by (initials)
☐ Equivalence	Exposure					
	KD60					
	Mortality					

Time Point (write an x)			
24 hour		120 hour	
48 hour		144 hour	
72 hour		168 hour	
96 hour			

SECTION C

	Net sample code	Piece number	Replicate	Exposure Start time hh:mm	Exposure End time hh:mm	No. mosq exposed	KD60	Alive	Dead
C		0	1						
C		0	2						
C		0	3						
C		0	4						
			1						
			2						
			3						
			4						

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IT LN DATA 002 V05 WHO Tunnel Test Data Sheet

To be completed in full in black or blue ink only. No pencil or tippex allowed. Return to study director within 7 days of completion /
Kumalizia kwa rangi nyeusi au bluu. Hakuna kutumia mfuto au penseli. Rudisha kwa mkuu wa kazi ndani ya siku saba baada ya kumaliza
SECTION A

Project Name **/ Jina la mradi:BIT|__|__|__|** **Study director Initials: |__|__|__|** **Sheet serial:** |__|__|__|__|

Date of exposure (dd/mm/yy) **/ tarehe ya kuachia (ss/mwe/mwa) |__|__|/|__|__|/|__|__|**

Test mosquito strain **/ aina ya mbu: |__|__|** Age of mosquitoes(d) **/ umri wa mbu(s): |__|-|__|** Temperature**/ Joto (°C) |__|__|.|__| Range 22-32 (°C)**

Tiny Tag Serial number: |____|____|____|____|____|_____| Relative humidity / **unyevunyevu** % **|__|__|.|__| Range 40-100**

Test/ **Jaribio**	Performed by (initials) / **imefanywa na nani**	Scored by (initials) / **imefanywa na nani**	Data entered by (initials) / **mwandishi nani**
Exposure/ **Muda wa kuweka mbu**			
Blood feeding/ **Wamekula damu**			
Mortality/ **Waliokufa**			

Test/ **Jaribio**	Start time hh:mm kuanza **saa:dakika**	End time hh:mm **kuamaliza saa:dakika**
Acclimatization/ **Kuweka mbu wazoee mazingira**		
Exposure/ **Muda wa kuachia**		

12 HOURS

				Releasing chamber- MOS (kuweka mbu)					**Bait chamber- BA (Upande wenye mtego- BA)**						
Tunnel Number	**Bait number**	**Net code**	**Total Exposed**	Fed dead / **Amekula na amekufa**	Unfed dead / **Hajala na amekufa**	Fed alive / **Mzima na amekula**	Unfed alive / **Mzima na hajala**	total MOS / **Jumla MOS**	Fed dead / **Amekula na amekufa**	Unfed dead / **Hajala na amekufa**	Fed alive / **Mzima na amekula**	Unfed alive / **Mzima na hajala**	total BA / **Jumla BA**	Temp/ **joto**	Humidity / **unyevunyevu**
1															
2															
3															
4															
5															
6															

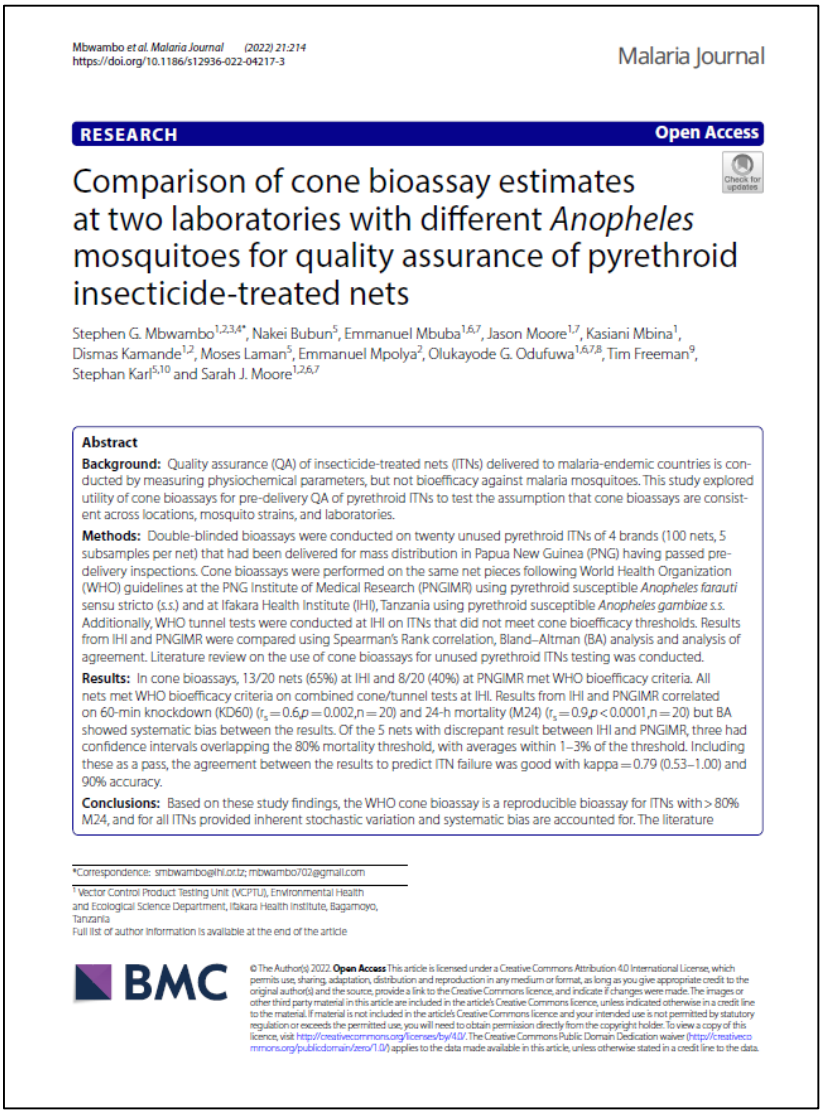
Control Fed; if <50 disregard test and repeat**/ kama mbu chini ya hamsini tu ndio wamekula katika tunnel ya control , rudia jaribio**

RESEARCH OUTPUTS

(a) Journal Paper

The research article has been published at BMC Malaria Journal 7th July, 2022. ID: MALJ-D-22-00026.

https://rdcu.be/cRcU1



Mbwambo et al. Malaria Journal (2022) 21:214
https://doi.org/10.1186/s12936-022-04217-3

Malaria Journal

RESEARCH Open Access

Comparison of cone bioassay estimates at two laboratories with different *Anopheles* mosquitoes for quality assurance of pyrethroid insecticide-treated nets

Stephen G. Mbwambo[1,2,3,4*], Nakei Bubun[5], Emmanuel Mbuba[1,6,7], Jason Moore[1,7], Kasiani Mbina[1], Dismas Kamande[1,2], Moses Laman[5], Emmanuel Mpolya[2], Olukayode G. Odufuwa[1,6,7,8], Tim Freeman[9], Stephan Karl[5,10] and Sarah J. Moore[1,2,6,7]

Abstract

Background: Quality assurance (QA) of insecticide-treated nets (ITNs) delivered to malaria-endemic countries is conducted by measuring physiochemical parameters, but not bioefficacy against malaria mosquitoes. This study explored utility of cone bioassays for pre-delivery QA of pyrethroid ITNs to test the assumption that cone bioassays are consistent across locations, mosquito strains, and laboratories.

Methods: Double-blinded bioassays were conducted on twenty unused pyrethroid ITNs of 4 brands (100 nets, 5 subsamples per net) that had been delivered for mass distribution in Papua New Guinea (PNG) having passed pre-delivery inspections. Cone bioassays were performed on the same net pieces following World Health Organization (WHO) guidelines at the PNG Institute of Medical Research (PNGIMR) using pyrethroid susceptible *Anopheles farauti* sensu stricto (s.s.) and at Ifakara Health Institute (IHI), Tanzania using pyrethroid susceptible *Anopheles gambiae* s.s. Additionally, WHO tunnel tests were conducted at IHI on ITNs that did not meet cone bioefficacy thresholds. Results from IHI and PNGIMR were compared using Spearman's Rank correlation, Bland–Altman (BA) analysis and analysis of agreement. Literature review on the use of cone bioassays for unused pyrethroid ITNs testing was conducted.

Results: In cone bioassays, 13/20 nets (65%) at IHI and 8/20 (40%) at PNGIMR met WHO bioefficacy criteria. All nets met WHO bioefficacy criteria on combined cone/tunnel tests at IHI. Results from IHI and PNGIMR correlated on 60-min knockdown (KD60) ($r_s = 0.6, p = 0.002, n = 20$) and 24-h mortality (M24) ($r_s = 0.9, p < 0.0001, n = 20$) but BA showed systematic bias between the results. Of the 5 nets with discrepant result between IHI and PNGIMR, three had confidence intervals overlapping the 80% mortality threshold, with averages within 1–3% of the threshold. Including these as a pass, the agreement between the results to predict ITN failure was good with kappa = 0.79 (0.53–1.00) and 90% accuracy.

Conclusions: Based on these study findings, the WHO cone bioassay is a reproducible bioassay for ITNs with > 80% M24, and for all ITNs provided inherent stochastic variation and systematic bias are accounted for. The literature

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(b) Convening Presentation

This work presented at second Raising the Floor on Nets Convening was held in Liverpool, UK 17-19th May, 2022. https://innovationtoimpact.org/raising-the-floor-on-nets-may-2022-convening/

(c) Poster Presentation

This work presented to the IHI Scientists, National Malaria control programs in Dodoma, Tanzania and Regional Health Management Team, Lindi, Tanzania at different time point.

Cone bioassay gives reproducible Bioefficacy estimates with different Anopheline mosquitoes and can be used for Quality Assurance of pyrethroid Insecticide Treated Nets

Stephen G. Mbwambo[1,2], Emmanuel Mpolya[2] and Sarah J. Moore[1,2]

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[2] Nelson Mandela Africa Institution of Science and Technology, Arusha, Tanzania

INTRODUCTION

The cone bioassay provides a simple evaluation of ITN bioefficacy, and its conditions and parameters are prescribed by World Health Organization (WHO). Yet, robust data comparing how the use of different mosquito species, across locations and laboratories may affect bioassay outcomes is needed. This study explored utility of a cone bioassay for pre-delivery quality assurance (QA) of pyrethroid ITNs.

MAIN OBJECTIVE

To explore utility of cone bioassay for pre-delivery QA of ITNs in two test facilities using different mosquito species to test the assumption that cone bioassays are consistent and reproducible across locations, mosquito strains, and laboratories.

MATERIALS AND METHODS

Double-blinded bioassay tests were conducted on twenty unused pyrethroid ITNs at Ifakara Health Institute (IHI), Tanzania against susceptible *An. gambiae* s.s and Papua New Guinea Institute of Medical Research (PNGIMR)-reference lab against susceptible *An. farauti* s.s.

Fig. 1

Cone bioassay results from both testing facilities were compared using correlation analyses, Bland and Altman methods comparison, and Cohen's kappa.

Fig. 2

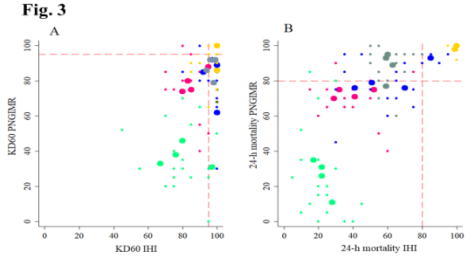
Experiment	*Bioassay test in IHI*	*Cone bioassay test in PNGIMR*
Number of ITNs tested	20 nets (100 net pieces)	20 nets (100 net pieces)
Mosquitoes exposed	20 per net piece (cone bioassay) 100 per net piece (tunnel tests)	20 per net piece (cone bioassay)
Experiment conditions	27±1°C 55% - 82% RH	28 ±4°C 53% - 71% RH
Mosquito species	Pyrethroid susceptible* *An. gambiae* s.s	Pyrethroid susceptible* *An. farauti* s.s
Mosquito age	3-5 days (cone bioassay) 5-8 days (tunnel tests)	2-5 days (cone bioassay)
WHO efficacy criteria	≥ 95%KD60 or ≥80% M24 (cone bioassay) ≥ 90% FI and/or ≥80% M24 (tunnel tests)	≥ 95% KD60 or ≥80% M24 (cone bioassay)

RESULTS

13/20 nets (65%) met cone WHO bioefficacy criteria at IHI and 8/20 (40%) at PNGIMR. All nets met WHO bioefficacy criteria on combined cone/tunnel tests.

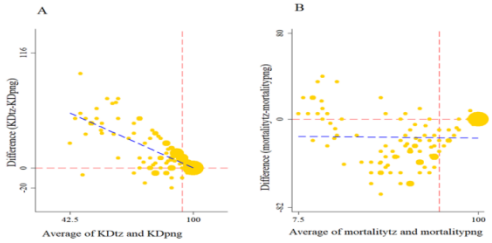
Cone bioassay results at IHI and PNGIMR correlated on 60-minute knockdown (r_s=0.6, p=0.002, n=20) and 24-hour mortality (r_s=0.9 p<0.000, n=20) **Fig.3**, but there was systematic bias between the results measured by Bland Altman **Fig.4**. The limits of agreement for both endpoints were wide: KD60 mean difference (limits of agreement) 15.5 (-25.4 to 56.5) and M24 -17.0 (-61.4 to 27.3).

Fig. 3



Of the 5 nets with discrepant result between IHI and PNGIMR, three had confidence intervals overlapping the 80% WHO mortality threshold, with averages within 1-3% of the threshold.

Fig. 4



The agreement between the results at IHI and PNGIMR to predict ITN failure was near perfect with kappa=0.79 (0.53-1.00) and 90% accuracy.

Fig. 5

		PNGIMR		
		Pass N (%)	**Fail N (%)**	**Total N (%)**
IHI	**Pass**	**12[a] (100)**	**2[c] (25)**	**14 (70)**
	Fail	**0[b] (0)**	**6[d] (75)**	**6 (30)**
	Total	**12 (60)**	**8 (40)**	**20 (100)**

a' and 'd' the number of nets agreed results at both testing facilities, 'b' and 'c' the number of nets with discrepant results between testing facilities

CONCLUSION AND RECOMMENDATIONS

WHO cone is a reproducible means to measure pyrethroid ITN bioefficacy using a combination of knockdown and mortality. In the absence of an alternative laboratory test, cone tests could assess the availability of active ingredients at the surface of the net where mosquitoes encounter it as part of pre-delivery QA.

We suggest the National Malaria Control Program (NMCP) in endemic countries to incorporate cone tests for ITNs bioefficacy check on pre-delivery inspections and/or prior to distribution.

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