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Modified who tunnel test for high through put evaluation of insecticide-treated nets considering the effects of hosts, exposure time and mosquito density

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MODIFIED WHO TUNNEL TEST FOR HIGH THROUGH PUT EVALUATION OF INSECTICIDE-TREATED NETS CONSIDERING THE EFFECTS OF HOSTS, EXPOSURE TIME AND MOSQUITO DENSITY

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ABSTRACT

The standard World Health Organization (WHO) tunnel test is a reliable laboratory bioassay used for "free flying" testing of Insecticide Treated Net (ITN) bio-efficacy. Multiple parameters affect the outcomes measured in tunnel tests. Therefore, a comparison of hosts, exposure time, and mosquito density against the current gold standard test (100 mosquitoes, animal bait and 12-hours exposure) was conducted following ITNs evaluation guidelines. A series of experiments were conducted in the WHO tunnel bioassay to evaluate the bio-efficacy endpoints, mortality at 24-hours (M24) and 72-hours (M72), blood feeding success (BFS), and blood feeding inhibition (BFI). The following parameters were evaluated: 1) baits (rabbit, membrane, human arm), 2) exposure time in the tunnel (1-hours vs 12-hours), and 3) mosquito density (50 vs 100 mosquitoes per test). Finally, an alternative bioassay using a membrane with 50 mosquitoes was compared to the gold standard bioassay. Resistant *Anopheles arabiensis* and susceptible *Anopheles gambiae* were used to evaluate Interceptor® and Interceptor®G2 ITNs. Similar trends in mortality and BFI were observed for both ITNs using the gold standard WHO tunnel test or alternative bioassays. Mortality and BFS were not statistically different when rabbits were the bait or when 50 or 100 mosquitoes of either strains used. No systematic difference was observed for the agreement by Bland and Altman's methods (B&A) with a mean difference 4.54% in blood feeding and 1.71% for M72. When comparing membrane with 50 mosquitoes and rabbit with 100 mosquito, no systematic difference was observed for the agreement with mean difference 9.06% for blood feeding and -5.44% for M72. These results demonstrate that WHO tunnel tests using rabbit bait run with 50 mosquitos' measures similar outcome compared to gold standard bioassay. In addition, using a membrane feeder with 50 mosquitoes is a potential replacement for the WHO tunnel bioassay with animal bait and merits further studies at other laboratories to corroborate these findings.

Jina la utafiti (Title)

"WHO Tunnel test" ilioboreshwa katika tathmini ya kuhakiki ubora wa vyandarua vilivyotiwa viua mbu kwa kuzingatia athari za chambo, muda, na idadi ya mbu.

Muhtasari (Abstract)

"WHO Tunnel test" ni njia ya uchunguzi wa kibaiolojia unaofanyika maabara kuhakiki ubora wa vyandarua vilivyotiwa viua mbu. Katika jaribio hili mbu wanaoruka hupita katika sampuli ya chandarua ili kufikia na kula damu ya chambo. Tulifanya utafiti wa kulinganisha vigezo mbadala dhidi ya vigezo vinavyotumika sasa kama ilivyoainishwa katika muongozo wa kupima ubora wa vyandaru vyenye viua mbu kwa lengo la kuboresha "WHO Tunnel test" ili iweze kufanyika kwa sampuli nyingi za vyandarua vyenye kiua mbu na kwa gharama nafuu zaidi. Tulifanya majaribio ya kuangalia matokeo ya kibiolojia ambayo ni kutambua (1) mbu waliokula damu na wasiokula damu na (2) mbu waliokufa saa 24 na saa 72 baada ya jaribio. Jaribio la kwanza tulichunguza ufanisi wa vigezo vifuatavyo; (1) aina ya chambo (sungura, kulisha kwa njia ya utando, na mkono wa binadamu). Jaribio la pili ni kutambua muda wa mbu kula damu (saa 1 dhidi ya saa 12). Jaribio la tatu ni kutambua idadi ya mbu (50 dhidi ya 100). Jaribio la nne, ni kulisha kwa njia ya utando na mbu 50 ikilinganishwa na sungura na mbu 100 (vigezo vinavyotumika sasa). Katika majaribio haya tulitumia mbu wa aina ya *Anopheles arabiensis* na *Anopheles gambiae* na vyandarua aina ya Interceptor® pamoja na Interceptor®G2. Matokeo ya mbu waliokufa na wasiokula damu kwa vyandarua aina zote yalikuwa sawa kwa njia inayotimika sasa "WHO Tunnel test" ikilinganishwa na njia ya kulisha damu kwa utando na mbu 100. Matokeo ya mbu waliokufa na waliokula damu yalikuwa sawa wakati sungura na mbu 50 au 100 walipotumika. Hakukuwa na tofauti kati ya njia ya sasa ikilinganishwa na njia mbadala kwa vigezo vya wastani wa mbu waliokula damu (4.54%) na mbu waliokufa saa 72 baada ya jaribio (1.71%). Pia, hakukuwa na tofauti kwenye wastani wa mbu waliokula damu (9.06%) na mbu waliokufa saa 72 (-5.44%) kati ya kulisha kwa utando na mbu 50 ukilinganisha na sungura na mbu 100. Utafiti huu umeonyesha kuwa ukitumia chambo sungura na mbu 50 tunapata matokeo sawa na vigezo vinavyotumika sasa. Pia, kutumia utando na mbu 50 kunaweza kufanikisha adhima ya kuacha kutumia chambo sungura, kuongeza idadi ya sampuli na kupunguza gharama ya kuhakiki ubora wa vyandarua kwa njia ya "WHO Tunnel test".

AUTHOR'S DECLARATION

I **Dismas Simwela Kamande**, hereby declare that, the dissertation submitted at the Institution for the requirement of the fulfilments of Master of Science in Public Health Research is my original work. The scholar's works, whenever used and included as the reference are acknowledged. I declare that, this dissertation was not submitted anywhere except Nelson Mandela African Institution of Science and Technology for academic qualification.

<u>13.08.2022</u>

Dismas S. Kamande   **Date**

STATEMENT OF COPYRIGHT

CERTIFICATION

The undersigned certify that they have read and hereby confirm that the dissertation entitled "Modified WHO tunnel test for high throughput evaluation of Insecticide-Treated Nets considering the effect of hosts, exposure time, and mosquito density" submitted by Dismas Simwela Kamande to Nelson Mandela African Institution of Science and Technology, Tanzania in fulfilment of the requirement for the award of Master of Science degree in Public Health Research is trustworthy work done under our supervision.

12.08.2022

Prof. Sarah J. Moore   **Date**

12.08.2022

Dr. Emmanuel Kaindoa   **Date**

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DEDICATION

I dedicate this work to my late father Daud M. Kamande and lovely mother Theresia Bartazary for raising and filling me with their love, teachings, prayers, and guidance. My dedication extends to all who have been influential to me in achieving this dream professional. Sincere dedication to my mentor and supervisor Sarah J. Moore, who has been committed with consistent support despite difficult moments. Lastly, my hard work with resilience and perseverance make this study complete timely.

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LIST OF ABBREVIATIONS

AIs	Active ingredient
B&A	Bland and Altman
BASF	Badische Anilin und Soda Fabrik
CO_2	Carbon dioxide
GTS	Global Technical Strategy
GMP	Global Malaria Program
GLMMs	Generalized linear mixed effects models
I- ACT	Ifakara Ambient Chamber Test
ICF	Informed consent form
IHI	Ifakara Health Institute
IRB	Institutional review board
ITNs	Insecticide treated nets
NIMR	National Institute of Medical Research
NMCP	National Malaria Control Program
NM-AIST	Nelson Mandela African Institution of Science and Technology
OR	Odds ratio
PBO	Piperonyl butoxide
PPF	Pyriproxyfen
WHO	World Health Organization
WHO-PQ	World Health Organization Pre-qualification
VCPTU	Vector Control Product and Testing

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Vector control in combinations with other interventions continues to offer effective prevention of mosquito-borne disease globally (WHO, 2021). Insecticide Treated Nets (ITNs) have been an extremely effective control measure (Pryce *et al.*, 2018) because they interrupt malaria transmission in two ways, by reducing mosquito blood feeding and also killing a proportion of mosquitoes that contact the nets (Birget, *et al.,* 2015; Koella, *et al.,* 2009). Since 2015, however, malaria control progress has stalled, with the COVID-19 pandemic in 2020 placing additional constraints on malaria control efforts. Despite this, ITNs remain the current cornerstone of global malaria control (WHO, 2021).

Pyrethroid insecticides are a recommended class for use in treating bed net. It reduces the number of bites that individuals sleeping under them receive even if the nets become old and torn, because pyrethroids inhibit mosquito flight and feeding responses (Carnevale, *et al.,* 1992; Chandre *et al.*, 2000). However, mass deployment of pyrethroid ITNs globally has led to widespread pyrethroid resistance with varying mechanisms observed in 82 countries (Cook *et al.*, 2018; Kleinschmidt *et al.*, 2018; WHO, 2021). To sustain the malaria control gains attributed to ITNs and to assist in reducing malaria by at least 90% by 2030 (WHO, 2021), several ITNs with different insecticide classes in combination with pyrethroids have been developed. These so-called "dual-insecticide ITNs'' afford non-neurotoxic modes of action with no cross-resistance (chlorfenapyr), reduced fecundity, and fertility pyriproxyfen (PPF), or increased susceptibilities to pyrethroids piperonyl butoxide (PBO) (Mosha *et al.*, 2022a). Randomized control trials have demonstrated greater malaria control using dual-insecticide ITNs compared to pyrethroid only nets in areas of high pyrethroid resistance, with pyrethroid containing PBO (Gleave *et al.*, 2021; Staedke *et al.*, 2020), or chlorfenapyr (Mosha *et al.*, 2022b). Operational research has indicated an additional public health benefit of chlorfenapyr (PATH, 2022) and pyriproxyfen (PATH, 2022) in combination with pyrethroid compared to pyrethroid-only ITNs.

New ITN products must demonstrate their continued effectiveness in the field for malaria control up to three years after deployment through biological efficacy testing against mosquito vectors (Hougard, *et al.,* 2003; WHOPES, 2013). The current WHO guidelines for ITN testing outline bioassays were designed to evaluate pyrethroidswith rapid neurotoxic action against exposed mosquitoes i.e. rapid incapacitation (knockdown), reduction in blood feeding, and killing within 24 hours post-exposure (WHOPES, 2013). This is different to dual AI ITNs like chlorfenapyr that requires the mosquito to be metabolically or physiologically active (as it is when encountering ITNs during host-seeking) to metabolize the parent molecule into the potent n-dealkylated form that elicits mosquito mortality (David, 2021). Mosquitoes are more metabolically active at night, when flying, host seeking or active during their typical circadian rhythms, for which the "free-flying" WHO tunnel test is a more appropriate bioassay (Kibondo *et al.*, 2022; Oxborough, 2015).

The WHO tunnel test is widely used to assess the bio-efficacy of ITNs under laboratory conditions. Despite predicting similar bio-efficacy of pyrethroid (Chandre, 2000) and chlorfenapyr ITNs (Kibondo *et al.*, 2022; Oxborough, 2015) to that measured in gold-standard experimental hut trials (Oxborough *et al.*, 2013), the tunnel test has several limitations (Massue *et al.*, 2019). Firstly, the animal baits (rabbit, guinea pig) used are non-preferred hosts for malaria mosquitoes, especially the highly anthropophilic Afrotropical vectors *Anopheles gambiae* and *Anopheles funestus*, also similar to opportunistic *Anopheles arabiensis* (Takken & Knols, 1999; Takken & Verhulst, 2013). Moreover, the use of animals has welfare concerns and is costly to ensure that animals maintained under veterinary supervision. Secondly, the bioassay time conducted overnight for (12-15 hours). Evidence shows *An. Arabiensis* interact with treated netting within the first 30 minutes of release (Parker *et al.*, 2017), thus prolonging exposure time could overestimate outcomes. Thirdly, the current tunnel test uses 100-mosquitoes per replicate, which is expensive for insectaries to produce. Owing to the significance of blood feeding in the life-cycle of the malaria mosquito as well as its importance for malaria transmission between human and mosquito hosts. It's an important component of vector control product testing. Membrane feeding has been widely deployed for evaluating topical mosquito repellents (Debboun, 2004), transmission-blocking drugs and vaccines (Vallejo, *et al.,* 2016), endoctocides (Smit, 2018) as well as for mosquito rearing (Awono-Ambene *et al.*, 2001; Damiens, 2013; Kaufman, 2014; N, 2017; Phasomkusolsil *et al.*, 2013; Robert, 1998; Timinao *et al.*, 2021). Moreover, the use of an artificial membrane has several advantages including reduced animal welfare or ethical concerns, reduced chance of accidental

disease transmission, simple logistics, and reproducibility (Ouédraogo, *et al.,* 2013; Kaufman, 2014; Phasomkusolsil *et al.*, 2013; Timinao *et al.*, 2021). Given the significance of host kairomones to encourage mosquito feeding, worn socks added to augment the attractiveness of the membrane to mosquitoes (Pates *et al.*, 2001) during host seeking process.

Multiple parameters including alternative hosts(Takken & Verhulst, 2013), mosquito density (Edman *et al.*, 1972), and duration of exposure to ITNs (Lines *et al.*, 1987) may affect the outcomes measured in tunnel tests. Therefore, the current thesis compared alternative hosts, exposure times, and mosquito densities against the current gold standard test (100 mosquitoes, animal bait, and 12-hours exposure) as outlined in the WHO ITN evaluation guideline (WHOPES, 2013) in an attempt to simplify the tunnel test to make it cheaper and higher throughput evaluation of large numbers of ITNs as needed for the bio-efficacy durability monitoring of chlorfenapyr ITNs that must be evaluated in "free-flying" bioassays ITN (Lissenden *et al.*, 2022).

1.2 Statement of the Problem

Evaluations of ITNs are performed to verify whether ITNs continue to maintain efficacy throughout their lifespan. ITN evaluations use WHO laboratory bioassays (i.e. cone, tunnel tests, and I-ACT) as well as experimental huts, and field trials in the community (WHOPES, 2013). Tunnel tests performs well in evaluating ITN bio-efficacy with key endpoints; (blood-feeding inhibition $\geq 90\%$ or mortality $\geq 80\%$) (Hougard, *et al.,* 2003). Currently, the tunnel test uses a live animal as bait (i.e. rabbit or guinea pig), exposure time (12-15 hours), and 100-mosquitoes density on evaluating ITN bio-efficacy (WHOPES, 2013). However, the bait used is not preferred for anthropophilic *Anopheles* mosquitoes (Takken & Knols, 1999; Vantaux *et al.*, 2014) and might overestimate the true effect on mortality and blood-feeding success or blood-feeding inhibition. Moreover, exposure time (12 hours) might overestimate the natural time of contact while testing one sample per replicate using 100 mosquitoes is expensive for insectary mosquitoes production. Further exploration of these parameters might allow the development of more cost-effective and allow a large sample size higher throughput when conducting efficacy and durability bio-efficacy bioassays of ITNs. Here, the current study investigated a modified WHO tunnel test for the evaluation of ITNs bio-efficacy by considering the effects of alternative baits, exposure time, and mosquito densities.

1.3 Rationale of the Study

The WHO tunnel test is a free flying test and has been shown to be a reliable bioassay for evaluating chlorfenapyr ITNs bio-efficacy (Oxborough *et al.,* 2015). In particular, the WHO tunnel is important as it allows evaluation of slow acting pro-insecticides (chlorfenapyr) that must be metabolized into the potent molecule in order to be efficacious. Importantly, tunnel tests predicts similar results to the experimental hut for both pyrethroid ITNs and chlorfenapyr ITNs (Jean-Marc Hougard, 2003). Durability monitoring of new and old ITNs requires cost-effective methods for evaluating ITNs because large numbers of samples need to be evaluated for precise estimate of efficacy results. Using alternative baits (membrane assay), reduced exposure (1 hour) and reduced mosquitoes (50 mosquitoes) may be means to make the tunnel bioassay cheaper and simpler. Here, three variations of WHO tunnel bioassay was investigated and compared to the standard WHO tunnel test method to determine if measure similar mortality and blood feeding outcomes when evaluating ITNs bio-efficacy.

1.4 Research Objectives

1.4.1 General Objective

To investigate modified WHO tunnel test protocols for higher throughput of evaluating ITNs bio-efficacy.

1.4.2 Specific Objectives

The study intended to achieve the following objectives:

(i)   To determine if alternative baits (membrane feeder and human arm) result in similar levels of mortality and blood feeding inhibition to the gold standard when evaluating ITNs bio-efficacy in the WHO tunnel test.

(ii)   To determine if reduced exposure time (1-hours vs 12-hours) results in similar levels of mortality and blood feeding inhibition to the gold standard when evaluating ITNs bio-efficacy in the WHO tunnel test.

(iii)   To investigate if mosquito density (50 vs 100 mosquitoes) results in similar levels of mortality and blood feeding inhibition to the gold standard when evaluating ITNs bio-efficacy in the WHO tunnel test.

(iv)   To investigate if alternative bioassay (50 mosquitoes-membrane feeder) results in similar levels of mortality and blood feeding inhibition to the gold standard (100 mosquitoes-rabbit bait) when evaluating ITNs bio-efficacy in the WHO tunnel test.

1.5 Research Questions

(i)   Can one hour exposure (1 hour) can be used instead of 12 hours exposure time?

(ii)   Can membrane baits measures similar outcome to gold standard baits?

(iii)   Can fifty mosquitoes (50) can be used instead of 100 mosquitoes?

(iv)   Can membrane feeder be used instead of rabbit as the bait?

1.6 Significance of the Study

Study findings demonstrate that a modified WHO tunnel test protocol can increase throughput in evaluating insecticide treated nets (ITNs), compared to current protocols. The alternative baits (membrane feeding) resolves animal welfare concern and make the bait reliable and shorten evaluation time as it does not require resting between experimental day. Reduced exposure time save time for evaluation and lower mosquito density might decrease cost for testing one sample of ITN. In addition, modified WHO protocol provide a relevant bioassay for biological durability monitoring of single and dual active ingredient ITNs with slow and fast acting mode of action.

1.7 Delineation of the Study

The study has a number of limitations, which need consideration when addressed in subsequent work. Firstly, experiments conducted in a single testing facility. A comparison of the alternative method in multiple laboratories is desirable to ensure the reproducibility of the methods with other mosquito strains. The low feeding success with the membrane technique needs to be overcome as clearly feeding success impacts mosquito mortality. Ideally, the membrane bioassay need improvement to consistently measure 50% mosquito feeding success at multiple testing facilities. Additionally, two different ITNs products both from the same manufacturer were used.

CHAPTER TWO

LITERATURE REVIEW

2.1 Impacts of ITNs on mosquito behavior and malaria control

Insecticide-treated nets (ITNs) are cost-effective tools that kill and interfere with the mosquito life cycle and control transmission of vector-borne disease globally. The ITNs provide physical protection by reducing vector-human contact, hence reducing the biting frequency and lowering malaria transmission (Malima, 2009; Malima *et al.*, 2008). Insecticide-treated nets kill mosquitoes that land on them, shortening their survival. Also they are less likely to survive the intrinsic incubation period of *Plasmodium* (Birget, 2015; Koella, 2009). ITN and use at a 50% rate in the community decreases the vectorial capacity (amount of malaria transmitted) of *An. gambiae* by greater than 90% (Le Menach *et al.*, 2007). In addition, with maximum coverage, the treated nets offer community protection once coverage, access, and use are high (>80%) (Hawley *et al.*, 2003; Magesa, 1991). The majority of the ITNs in public use are either coated or incorporated with pyrethroid insecticides. Some of these insecticides have different modes of action ranging from killing, effects on fecundity as well as excito-repellent properties that divert mosquitoes from the protected population and prolong the feeding cycle. However, repellents may not always possess the ideal properties because they can shift mosquitoes to an unprotected population, thereby increasing transmission intensity. Regardless of the loss in its efficacy and effectiveness over time, ITNs still demonstrate impacts on reducing mosquito survival and transmission intensity in the at-risk population.

2.2 Variation of bioassay method in testing ITNs bio-efficacy

Bio-efficacy testing of ITNs remains one pillar in the effort of generating evidence of on new generation or new ITNs. The WHO uses findings from key vector control stakeholders (i.e. NMCPs, Research institutions) to recommend ITNs for use (WHO, 2017; WHOPES, 2013). ITNs must demonstrate their efficacy in different phases before recommendations are made for public use, through WHO laboratory bioassays: cone and tunnel (Phase I), small field trial i.e. experimental hut (Phase II) and community durability trial (Phase III) (WHOPES, 2013). The WHO cone bioassay is the first and most important method that evaluates the bio-efficacy of new

2.2.1 WHO Cone bioassay and its limitations

Cone bioassays are designed to evaluate the contact toxicity of ITNs against malaria vector mosquitoes. In cone tests, bio-efficacy cut-off endpoint thresholds after a three minute exposure are ≥ 80% mortality at 24 hours or ≥95% knockdown after one hour of holding exposed mosquitoes (WHOPES, 2013). However, the cone test is liable to limitations: mosquitoes can avoid treated surfaces during the test for the cone tests this could overestimate mortality due to forced contact (Massue *et al.,* 2019). Furthermore, this test is not relevant screening methods for slow-acting pro-insecticides (chlorfenapyr) the chemical that activated after mosquito contacts net surface.

2.2.2 WHO tunnel bioassay and its limitations

In situations where nets fail to meet the bio-efficacy criteria in the WHO cone tests, these nets can then be subjected to WHO tunnel tests. The WHO tunnel is made of glass and it uses live animals (guinea pigs or rabbits) as bait and treated netting. Tunnel test bioassay are conducted at night to simulate the night phase for host-seeking malaria vector. Moreover, tunnel outcome measures allow a better observation of mosquito avoidance behavior and toxicity of excito-repellent and pro-insecticides like chlorfenapyr (Oxborough, 2015; WHOPES, 2013). In addition, the tunnel test predicts the results obtained in the experimental hut trial (Kibondo *et al.*, 2022; Richard, 2015). The thresholds for this test are of mortality ≥ 80% and feeding inhibition ≥90% (WHO, 2013). However, using rabbit to measure host-seeking behavior underestimate feeding success, particularly for *An. gambiae, An. funestus,* and opportunistic *An. arabiensis* (Takken & Knols, 1999; Takken & Verhulst, 2013; Vantaux *et al.*, 2014). This is because these anthropophilic species prefer feeding on a human. In addition, the use of live animals for testing is raise animal welfare concern and the cost of caring for the animals can be challenging.

2.3 Essential of tunnel tests for new generation of ITNs

The ongoing spread of insecticide resistance to the recommended active ingredient (i.e. pyrethroids) in malaria vectors if not controlled might compromise the efficacy of vector control tools (Cook *et al.*, 2018). For this reason, chlorfenapyr (CFP) insecticides from the pyrrole class with a different mode of action were developed. The CFP is a pro-insecticide with the mode of action that hinders the mosquito's ability to produce energy at a cellular level (R, 2007). Moreover,

the mosquito ingests the chlorfenapyr and metabolized to second de-alkylated form that is insecticidal to the vector (Michael, 2021; R, 2007). It takes a minimum post-exposure time of 48-72 hours to kill mosquitoes as the molecule is metabolized into the insecticidal form over time (R, 2007). In laboratory and field setting trials, CFP alone or a mixture with pyrethroids shows greater efficacy and effectiveness in controlling pyrethroid-susceptible and pyrethroid-resistant mosquitoes (Mosha *et al.*, 2022a; Mosha *et al.*, 2008; N'Guessan *et al.*, 2014; Raghavendra *et al.*, 2011). However, screening slow-acting insecticides (i.e. CFP) using forced contacts bioassay (i.e. cone) might underestimate its killing effects because the mosquitoes are not metabolically active (Oxborough *et al.*, 2015). Here, the tunnel test is a relevant screening method because mosquitoes are free flying and host seeking (Oxborough, 2015).

2.4 Why host is needed for the WHO tunnel test

The mosquito life cycle and malaria transmission depend on the interaction between a host and the vector. The response of the vector to insecticide toxicity and other properties (i.e. repellent and irritant) is associated with host availability in behavior bioassays (Hossain, 1989; Pates *et al.*, 2001). This demonstrates that in the presence of hosts, host-seeking mosquitoes search for a blood meal as observed in household settings which encourages them to contact the ITN acting as a barrier between the mosquitoes and hosts. The WHO tunnel bioassay uses live animals (rabbits or guinea pigs) as host/bait when evaluating ITN efficacy (WHOPES, 2013). However, using a rabbit might attribute to some ethical concern, led to housing, and maintenance expenses, and can lead to accidental zoonosis infection (Kaufman, 2014; Massue *et al.*, 2019), thus requiring the development of alternative comparable host. Over the years, membrane feeding as an alternative host was used in drug and vaccine studies (Awono-Ambene *et al.*, 2001; Diallo *et al.*, 2008; Smit, 2018; Vallejo, 2016). Studies comparing different blood sources showed similar mosquito blood feeding compared to direct feeding (Damiens, 2013; N, 2017). Membrane feeding broadly continues to be a reliable means of mosquito feeding in colony maintenance in insectaries (Damiens, 2013; Phasomkusolsil *et al.*, 2013). Using colonies that have been adapted to feed on the membrane might yield high feeding success. Mosquitoes use visual, olfactory, and thermal cues to detect and locate a host for a blood meal (Dekker *et al.*, 2001; Takken & Knols, 1999; van Breugel *et al.*, 2015). The long, medium and short-range attraction mediated by different host cues influences free-flying mosquito responses toward the host (Lorenz *et al.*, 2013; van Loon *et al.*,

2015). Furthermore, mosquitoes are attracted by carbon dioxide at long distances while they distinguish between hosts at shorter distances by sweat and heat (Takken & Knols, 1999; van Loon *et al.*, 2015). In investigating odor preference in response to the host, a worn sock's scent attracts more mosquitoes than synthetic blends (Mburu *et al.*, 2017; Mukabana *et al.*, 2002; Smallegange *et al.*, 2011). Membrane feeding does not depend on the odor to influence mosquito feeding. In addition, membrane feeding recommended for use in disease-endemic areas where mosquitoes are at high risk of disease transmission (André Lin Ouédraogo, 2013; Kaufman, 2014).

2.5 Durability monitoring and durability bio-efficacy monitoring

Durability monitoring is a vital component in understanding the functional lifetime of ITNs under operational conditions (WHOPES, 2011). It measures ITNs integrity, survivorship, and bio-efficacy of old nets to demonstrate insecticidal composition and user behavior in sustaining net quality. Understanding the functional lifetime of ITNs by identifying factors that hinder durability and laboratory criteria is significant to maintain net quality (WHOPES, 2011). Durability monitoring activities provide information on product performance in a particular setting for guidance in planning, procurement, and replacement of nets (Randriamaherijaona *et al.*, 2015). ITNs as long-lasting insecticidal nets are considered to retain their biological potency after standard 20 washes and last for 3 years under field setting (WHOPES, 2011). Based on this assumption, the life span of ITNs is uniform regardless of AIs and locations. For bio-efficacy in durability monitoring, the cone test is best for screening ITNs with contact neurotoxins insecticides like pyrethroids only or pyrethroids with PBO. For the slow-acting pro-insecticides and other pyrethoid mixture ITNs, free flying bioassays such as tunnels tests with modification in some parameter is critical for baseline and durability bio-efficacy monitoring. For durability monitoring, 30 nets samples were recommended for bio-efficacy testing (WHOPES, 2011). For precise estimates of bio-efficacy results, large samples size is needed for the durability monitoring.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Bioassays were performed at the Vector Control Product Testing Unit (VCPTU) facility located at the Bagamoyo branch of Ifakara Health Institute (IHI), Tanzania (6.446ºS and 38.901ºE).

3.2 Description of Investigational ITNs

Interceptor® is made from 100-denier polyester coated with 200 mg/m² alpha-cypermethrin and Interceptor® G2 is made of 100-denier polyester coated with a mixture of 200 mg/m² chlorfenapyr and 100 mg/m²alpha-cypermethrin. Both net brands are manufactured by BASF, Germany. Safi Net made of polyester, manufactured by A to Z Textile Mills, Tanzania was used as a negative control to monitor the quality of the bioassay. The study included the following arms: (a) unwashed Interceptor®, (b) Interceptor® washed 20 times, (c) unwashed Interceptor® G2,(d) Interceptor® G2 washed 20 times, 5) negative control – Safi net. Five samples per net were cut and samples were washed twenty times according to a protocol adapted from the standard WHO washing procedure (Lissenden *et al.*, 2022) using 20g/liter palm soap (Jamaa brand). The interval of time used between two washes (i.e. regeneration time) was 1 day for both Interceptor® G2 and Interceptor® ITNs (Table 1).

3.3 Mosquitoes

Pyrethroid-resistant *Anopheles arabiensis* (Kingani strain, established 2017) and pyrethroid susceptible *Anopheles gambiae* (Ifakara strain, established 1996) were used in this study. *An. arabiensis* (Kingani) is metabolic-resistant and expresses the upregulation of cytochrome p450s, with 14% mortality upon exposure to WHO 1x discriminating dose of alpha-cypermethrin that was reversed by piperonyl butoxide (PBO) pre-exposure, reconfirmed before this study was initiated. *An. gambiae* s.s. (Ifakara) is fully susceptible to selected insecticide classes at 1x WHO discriminating doses, reconfirmed before this study was initiated. The mosquito colony was maintained according to MR4 Guidelines(Kaufman, 2014) at 27 ± 2 °C and 40%–100% relative humidity, with an ambient (approximately 12:12) light–dark cycle. The colony was maintained on

a Tetramin fish food for larvae, 10% glucose for adults. Females were offered cattle blood in a membrane feeder or were offered a human arm as a blood source. Mosquitoes were 5–8 days old, nulliparous, sugar starved for eight hours, and acclimatized to the test room for an hour before the experiment (Table1). As VCPTU do not have resistant *An. gambiae* in the colony, we used metabolic resistant *An. arabiensis* instead. Since the bioassay measured contact toxicity, it was deemed that the mechanism for resistance was more critical than the species used for the evaluation.

Table 1: Experimental setup

Experiment	1	2	3		4
Factor	Host/Baits	Exposure Time	Mosquito Density		Replacement of Rabbit
Comparison	Human or membrane vs. rabbit with 100 mosquitoes	1 h vs. 12 h for human or membrane (within host)	50 vs 100 mosquitoes using rabbit		Rabbit with 100 mosquitoes vs. membrane with 50 mosquitoes
ITNs arms	Interceptor® G2 Unwashed Interceptor® G2 Washed 20x Interceptor® Unwashed Interceptor® Washed 20x Negative control		Interceptor® G2 Unwashed Interceptor® G2 Washed 20x Negative control	Interceptor® Unwashed Interceptor® Washed 20x Negative control	Interceptor® G2 Unwashed Interceptor® G2 Washed 20x Interceptor® Unwashed Interceptor® Washed 20x Negative control
Replicates per arm per comparison	5		15	15	15
Total replicates	75	100	90	90	150
Number of nights	15	10	10	10	16
Mosquitoes exposed	100		100, 50		100, 50
Host/bait	Rabbit, Human, Membrane	Human, Membrane	Rabbit		Rabbit-100, Membrane-50
Exposure time	12 h	12 h / 1 h	12 h		12 h
Mosquito species	*Anopheles arabiensis*		*Anopheles arabiensis*	*Anopheles gambiae*	*Anopheles arabiensis*
Primary Outcomes	Blood feeding success (BFS), 24-h mortality (M24), 72-h mortality (M72)				
Additional Outcome	Blood feeding Inhibition (BFI)				

3.4 The Standard WHO tunnel test procedure

WHO tunnel tests were conducted following WHO guidelines (WHOPES, 2013; Fig. 1A). The tunnel is divided into two chambers separated by a netting sample that has been deliberately holed with 9 small (1cm) holes through which the mosquitoes must pass to reach the bait. In the short chamber, the bait was placed. In the long section, 100 unfed female mosquitoes aged 5–8 days were released at 1900 hrs. The tunnel was covered with a black cloth and left overnight. The following morning between 0700 hours and 0900 hours mosquitoes were removed from the tunnel using an aspirator. Mosquitoes were scored as alive fed, alive unfed, dead fed, or dead unfed in each chamber and put into a separate paper cup for most exposure mortality monitoring. Mosquitoes were supplied with access to a 10% sugar solution ad libitum and then scored for post-exposure delayed mortality at 24- and 72- hours. The experiment and post exposure holding was conducted at a temperature of $27 \pm 2°C$ and relative humidity of $80\% \pm 10$. For the experiment to be considered valid the following thresholds were used: control 24-hour mortality $\leq 10\%$ in all experiments and blood feeding success $\geq 50\%$ with experiments using the rabbit bait (WHOPES, 2013).

Figure 1: WHO tunnels for comparison of baits/hosts

Figure shows conduct of standard(A) WHO Tunnel with the bait chamber to the left of the picture and mosquitoes being placed into the longer end of the chamber; (B) Rabbit- in Experiments 1-4 (C) Hemotek® membrane – in Experiment 1&4 (D) Human arm – in Experiment 1.

3.5 Bait used and preparation

The following are the baits used and their preparation;

(a) Rabbit: Three groups of five healthy rabbits were used. Rabbits were maintained under veterinary supervision. The rabbit was shaved on its back to allow the mosquitoes to feed. The rabbit was gently restrained in a mesh tube that was suspended in the short section of the WHO tunnel throughout the 12-h experiment (Fig. 1B).

(b) Membrane feeding: AHemotek® membrane feeder (SP-6 System, Hemotek Ltd., Blackburn BB6 7FD, UK) was used. Two membrane feeders were placed on top of the "bait chamber" of each tunnel (Fig. 1C). Each feeder reservoir contained 3 ml of cow blood covered by a stretched parafilm membrane and tightened with an o-ring to prevent any leakage. Cow blood was obtained from cattle maintained under veterinary supervision at VCPTU and was stored for up to two weeks at 4–8 °C in heparinized tubes. Socks worn by the investigator (DK) for 8 h on the day of testing were stretched across the surface of the membrane feeder reservoir to provide host kairomones and increase mosquito attraction to the feeder. The Hemotek® was switched on 10 minutes before the experiment. The temperature of the feeder was set at 37–39 °C throughout the 12-h experiment.

(c) Human arm: Five healthy male volunteers conducted arm feeding by inserting their arms into the bait short section of the tunnel (Fig. 1D). Before testing, their arms were washed with water. The volunteers were non-smokers and did not drink alcohol or use perfumed lotions during the experimental period. The experimental time for arm feeding was 1 h to allow for standardized evaluation and to minimize volunteer discomfort. Previous work has shown that 30 minutes of exposure resulted in high blood feeding (Timinao *et al.*, 2021).

3.6 Study design

Series of experiments were comparative bioassays were conducted with a minimum of 5 replicates per net type, per permutation (Table 1). A total of sixty one experimental nights were run between

March 2021 and February 2022. All procedures for preparation, release, collection, and mosquito scoring were performed as per the standard WHO tunnel test procedure (WHOPES, 2013) (Fig. 1A) outlined above with the factors of interest (bait, exposure time, and density) varied (Table 1). The endpoints measured were blood feeding success (BFS) or blood feeding inhibition (BFI), mortality at 24-h (M24), and mortality at 72-h (M72).

3.6.1 The impact of bait/host

The bio-efficacy of unwashed and 20 times washed Interceptor®G2 and Interceptor® ITNs was tested using 100 pyrethroid resistant *An. arabiensis* per replicate with membrane, human arm, and rabbit bait (Fig.2A). Mosquitoes were left in the tunnel for 12 hours overnight and BFS, M24, and M72 endpoints were evaluated. Five samples for each ITN type (Interceptor®G2 and Interceptor®) and condition (washed and unwashed) for each host type were evaluated using five tunnels. One control and four treatments i.e. one per net type and condition were conducted each night for 15 nights with each bait (membrane, human, and rabbit) evaluated for five nights each. Each bait type was tested on different nights to allow independent comparison of each bait in the absence of competing host kairomones.
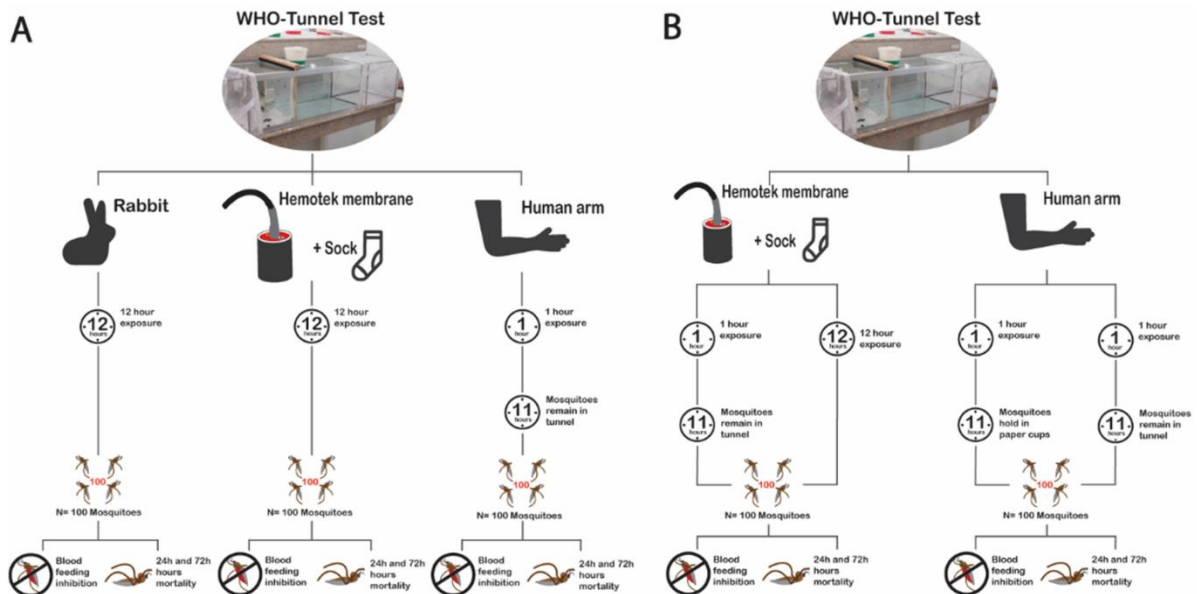
Figure 2: Flow chart of experimental procedure

Figure shows experiment 1 (A) impact of baits and experiment 2 (B) effects of exposure time 12 hours vs 1 hour on WHO tunnel test outcomes.

3.6.2 The impact of exposure time

The bio-efficacy of unwashed and washed 20 times Interceptor®G2 and Interceptor® was tested using 100 pyrethroid resistant *An. arabiensis* per replicate with either a human arm or membrane bait (Fig.2B). When investigating 1 hour exposure, mosquitoes were challenged to ITNs for only 1 hour in a human arm or membrane then removed from the tunnel and placed in holding cups with access to sugar for 11 hours overnight. For the 12 hour exposure, the human arm was only available for 1 hour followed by 11 hours with the mosquitoes left in the tunnel while the membrane was available to mosquitoes in the tunnel throughout the 12 hours of exposure. In both tests, the BFS, M24, and M72 endpoints were evaluated. Five samples for each ITN type (Interceptor®G2 and Interceptor®) and condition (washed or unwashed) were tested using five tunnels in each baits and exposure times with 5 replicates per treatment over 10 night of evaluation conducted in combination. The 1 hour and 12 hours of exposure were conducted on the same night for either the membrane or the human arm. Each bait type was tested on different nights to allow independent comparison of each bait in the absence of competing host kairomones.

3.6.3 Effects of mosquito density on the bio-efficacy measurement of blood feeding inhibition and mortality at 24/72hrs

The effect of mosquito density on bio-efficacy measurements of BFS, M24, and M72 endpoints was evaluated in the WHO tunnel using 50-mosquitoes compared to the standard 100-mosquitoes (Fig.3A). Experiments were conducted following standard procedures with 12 hours of exposure and continuous access to a restrained rabbit. For this, two species were used: pyrethroid resistant *An. arabiensis* tested for the pyrethroid and chlorfenapyr Interceptor®G2 (unwashed or washed 20 times) and pyrethroid susceptible *An. gambiae* for the pyrethroid only Interceptor® ITN (unwashed or washed 20 times). A total of seven tunnels (one control, 3 with unwashed, and 3 with washed ITNs) per night were run with 15 replicates conducted per net condition for each density. Each strain and density (Table1) was evaluated in a separate 5-night block. This was done to ensure fitness of mosquitoes used, as the experiments were conducted at a time when the mosquito colony was under pressure from multiple evaluations. Susceptible strain was selected to

present the general susceptible strain also to into existing evidence the performance of pyrethroid ITNs (Interceptor®). While metabolic resistance strain represent large percentage of resistance mechanism to mosquito population in endemic areas and how dual ITNs (Interceptor®G2) can offer maximum protection.
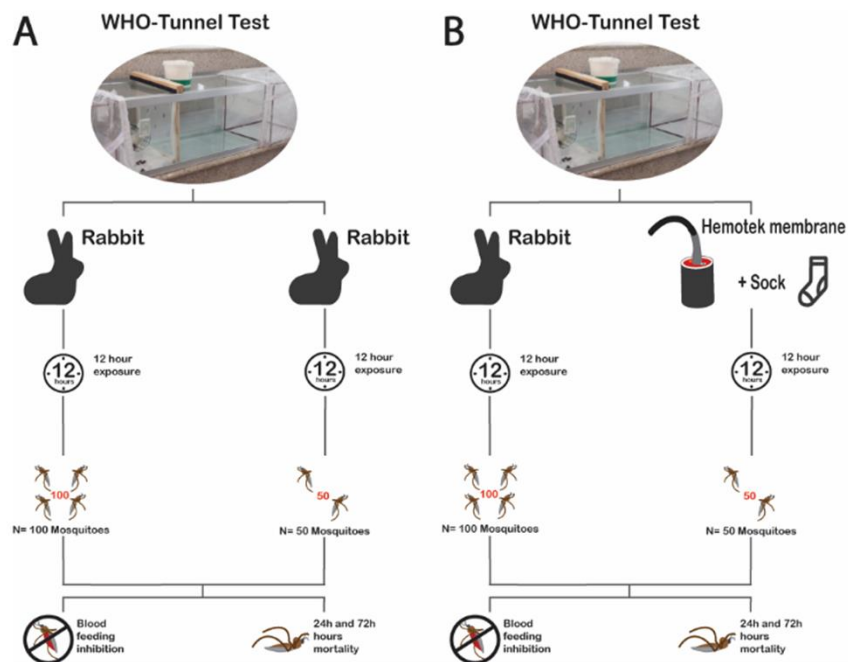
Figure 3: Flow chart of experimental procedure

Figure shows experiment 3(A)-Effects of mosquito density 100 vs 50 and experiment 4 (B)-Possibility to replace 100-rabbit bioassay with 50-Hemotek membrane.

3.6.4 Possibility to replace standard bait (rabbit) with the membrane assay

To determine if the rabbit can be replaced with the membrane assay as the bait, the bio-efficacy measurements of BFS, M24, and M72 endpoints were evaluated in the WHO tunnel with 12 hours of exposure using 50-membrane and 100-rabbit (gold standard) with resistant *An. arabiensis* mosquitoes (Fig.3B). The same procedure was used for all four treatment arms of Interceptor® and Interceptor® G2 ITNs, unwashed or washed 20 times (Table1). For the membrane, a total of 5 tunnels (one control and four treatments, one for each of unwashed or 20-times washed Interceptor® or Interceptor® G2) per night, and for the rabbit, nine tunnels (one control and eight treatment, two for each of unwashed or 20-times washed Interceptor® or Interceptor® G2) per

night, with 15 replicates per arm for each assay were conducted. Different baits were run on separate nights to allow independent comparison of each bait in the absence of competing host kairomones.

3.7 Data management and analysis

3.7.1 Sample size and power

A sample size calculation for generalized linear mixed effects models (GLMMs) through simulation (Johnson *et al.*, 2014) in R statistical software 3.02 https://www.r-project.org/ was performed for the I- ACT and experimental huts. For the I-ACT, to detect a 10% effect difference between the nets, simulations were performed using an estimated mosquito mortality of 80% for unwashed Interceptor® G2 and 70% for unwashed Interceptor®, and 10% for SafiNet® (deliberately holed). The power estimated was more than 90% based on estimates from previous studies conducted in the same setting: mean mortality of 81.5% for WHO tunnel test with an assumed daily variation of 0.5 and 15 replicates per arm (Kibondo *et al.,* 2022).

3.7.2 Statistical Analysis

Data were collected using standard paper forms and double entered into an Excel spreadsheet, cleaned, and imported into STATA 16.1 (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) for analysis. Descriptive statistics were used whereby mean percentage mortality at 24-hours (M24) or 72-hours (M72) or blood feeding success (BFS) or blood feeding inhibition (BFI) with their 95% Confidence Intervals (CI) were calculated. Multivariable mixed logistic regression with a binomial link was conducted with fixed effects for the exposure of interest, adjusting for ITN condition and mosquito species, with day as a random effect to account for daily variability in environmental conditions and mosquito batch variability. Model fit was checked by testing of model residuals. To estimate the superiority of Interceptor®G2 over Interceptor® with resistant mosquitoes, the same regression was used by comparing gold standard 100-rabbit to 50-membrane on M72 and BFS endpoint. In addition, Bland and Altman (Bland & Altman, 1999) methods were used in estimating the agreement in outcomes M24, M72 and BFS measured by assays: (a) membrane vs rabbit, (b) 100 vs 50 mosquitoes and (c) 100-rabbit vs 50-membrane.

3.8. Ethical Approval and Human arm feeding protection

To protect human participants, several procedures are routinely undertaken in the laboratory. Anybody who works in the insectary of blood feeds mosquitoes (including the participants) is screened weekly for malaria parasites using malaria rapid tests (SD bioline). Mosquitoes used in the experiments were nulliparous. Therefore, participants were not at risk of malaria infection. Ethical approval was obtained from the IHI Institutional Review Board (*IHI/IRB/No25-2021*) and the National Institute of Medical Research (*NIMR/GQ/R.8a/Vol.IX/3893*). Permission to publish was granted by the Tanzania National Institute of Medical Research letter with ref *NIMR/HQ/P.12 VOL XXXIV/39*.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Experimental validity

In all the bioassays conducted, control mortality at 24hours was <10% and at 72hours was <13%. Feeding success was ≥50% in both the human arm and the rabbit controls and was <23% in the membrane control (Appendix 1).

4.1.2 The impact of baits/hosts

The bait used affected both feeding and mortality endpoints measured. The membrane measured similar mortality and lower blood feeding success than the rabbit. The human arm measured lower mortality and higher blood feeding success than the rabbit.

The difference in the odds of mosquito mortality at 24-hours (M24) or 72-hours (M72) and blood feeding success (BFS) for 100 pyrethroid resistant *Anopheles arabiensis* exposed to Interceptor® and Interceptor® G2 with either a rabbit, human arm or membrane feeder as bait.

Table 2: Impact of bait on mortality and blood feeding adjusted for the net condition

	BFS			M24			M72		
	%(95% CI)	OR(95%CI)	P-value	%(95% CI)	OR(95%CI)	P-value	%(95% CI)	OR(95%CI)	P-value
Control									
Rabbit	64.8 (51.2- 78.3)	1		3.8 (0.8-6.8)	1		7.7 (5.1 - 10.3)	1	
Membrane	22.8 (10.4-35.1)	0.16 (0.14-0.20)	< 0.001	6.8 (5.9 - 7.6)	1.83 (1.22-2.75)	0.004	8.9 (8.3 - 9.5)	1.16 (0.84-1.59)	0.366
Human arm	74.4 (67.9-80.8)	1.59 (1.25-2.02)	< 0.001	6.4 (4.9 - 7.8)	1.71 (1.05-2.77)	0.030	11.7 (9.0 - 14.4)	1.58 (1.11-2.26)	0.012
Treatment									
Rabbit	6.6 (2.2-11.0)	1		49.7 (36.4 - 62.9)	1		66.1 (55.3 - 76.9)	1	
Membrane	4.6 (1.5-7.7)	0.34 (0.28-0.48)	< 0.001	46.5 (35.7 - 57.3)	0.90 (0.79-1.02)	0.086	67.2 (57.0 - 77.3)	1.07 (0.93-1.22)	0.352
Human arm	55.9 (49.1-62.7)	9.81 (8.25-11.67)	< 0.001	29.3 (22.1 - 36.5)	0.42 (0.37-0.48)	< 0.001	37.3 (29.7 - 45.0)	0.31 (0.27 - 0.35)	< 0.001

*Mosquitoes were exposed for 12 hours. Data presented are mean proportion (%) with 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% confidence interval (95% CI) adjusted for net type and condition.

Mosquito mortality at 24 hours was not significantly different between the rabbit and membrane (OR: 0.90, 95%CI: 0.79-1.02, p=0.086) and was significantly lower using the human arm (OR: 0.42, 95%CI: 0.37-0.48, p< 0.001) compared to the rabbit (Table 2). Mosquito mortality at 72 hours (M72) was not significantly different between the rabbit and membrane (OR: 1.07, 95%CI: 0.93-1.22, p=0.352) and significantly lower using the human arm (OR: 0.31, 95%CI: 0.27-0.35, p< 0.001) compared to the rabbit (Table 2). Mosquito feeding success was significantly lower using a membrane (OR: 0.34, 95%CI: 0.30-0.39, p<0.001) and significantly higher using a human arm (OR: 9.81, 95%CI: 8.25-11.67, p< 0.001) compared to the rabbit (Table 2). The same trends in mortality and blood feeding inhibition (BFI) were observed for both Interceptor® ITN and Interceptor®G2 (Fig.4). Higher blood feeding resulted in lower mortality (Appendix 4), which will explain the lower mortality measured with the human arm, which also has substantially higher blood feeding success. Therefore the human arm could not be considered for further evaluation. Between membrane and rabbit with 100 mosquitoes per replicate, no systematic difference in blood feeding and mortality was observed for agreement by Bland and Altman methods (Appendix 5). The mean difference was 6% (-10.81-23.01) for blood feeding success and -1.09% (-72.91-70.73) for M72.
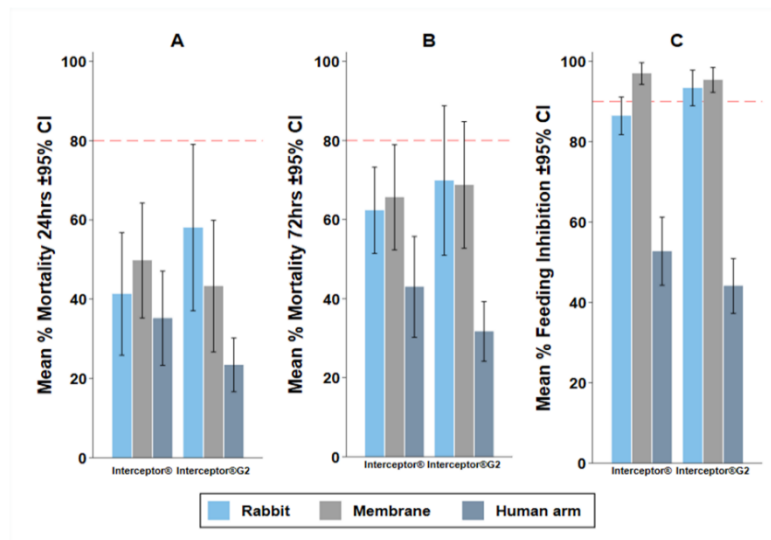
Figure 4: Mean and confidence Interval of mortality and blood feeding inhibition for resistant a*nopheles arabiensis*

Figure shows WHO tunnel outcomes (A) mortality 24-hours post exposure, (B) mortality 72-hours post exposure and (C) Blood feeding inhibition for Interceptor® and Interceptor®G2 nets with 100 pyrethroid-resistant Anopheles *arabiensis*mosquitoes using rabbit, Hemotek® membrane feeders and Human arm as bait in the WHO tunnel bioassay. The red dashed line depicts WHO minimum bio-efficacy criteria of ≥ 80% mortality and ≥ 95% blood feeding inhibition.

4.1.3 Impact of exposure time on mortality and blood feeding

Increasing the time that mosquitoes are left in the tunnel from 1 hour to 12 hours increased mortality with either the human arm or the membrane (Table 3). With the membrane bait, longer exposure significantly increased both the odds of mortality at 72 hours (OR: 2.30, 95%CI: 2.02-2.62, p=0.001) and odds of blood feeding (OR: 1.55, 95%CI: 1.08-2.22, p=0.017). Similarly in the human arm, the longer exposure significantly increased the odds of mortality at 72 hours (OR: 1.66, 95%CI: 1.45-1.90, p=0.001) while the effect of exposure time on blood feeding success could not be measured since the human arm was only available for one hour (Fig.5). The time that mosquitoes are left in the tunnel overnight is a significant factor in mosquito mortality and should always be recorded and reported.

The difference in the odds of mosquito mortality at 24 hours (M24) or 72-hours (M72) and blood feeding success (BFS) for 100 pyrethroid resistant *Anopheles arabiensis* exposed to Interceptor® and Interceptor® G2 with either a human arm or membrane feeder as bait*.

Table 3: Impact of exposure time on mortality and blood feeding adjusted for the net condition

Assays	BFS			M24			M72		
	% (95%CI)	OR (95%CI)	P-value	% (95%CI)	OR (95%CI)	P-value	% (95%CI)	OR (95%CI)	P-value
Membrane									
1hr-exposure	1.2 (0.1-2.3)	1		24.7 (17.0-32.4)	1		38.9 (26.5-51.2)	1	
12hr-exposure	4.6 (1.5-7.7)	1.55 (1.08-2.22)	0.017	43.3 (25.9-60.6)	1.66 (1.46-1.89)	< 0.001	68.8 (52.0-85.5)	2.30 (2.02-2.62)	< 0.001
Human arm									
1hr-exposure		NA		20.3 (17.7-22.8)	1		31.1 (26.1-36.1)	1	
12hr-exposure		NA		35.2 (22.7-47.6)	2.26 (1.93-2.64)	< 0.001	43.0 (29.6-56.3)	1.66 (1.45-1.90)	< 0.001

*Mosquitoes were exposed for either 1 hour before being removed from the tunnel and placed in holding cups with access to sugar or left overnight in the tunnel for 12 hours. Data presented are mean proportion (%) with 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% confidence interval (95% CI) adjusted for net conditions.
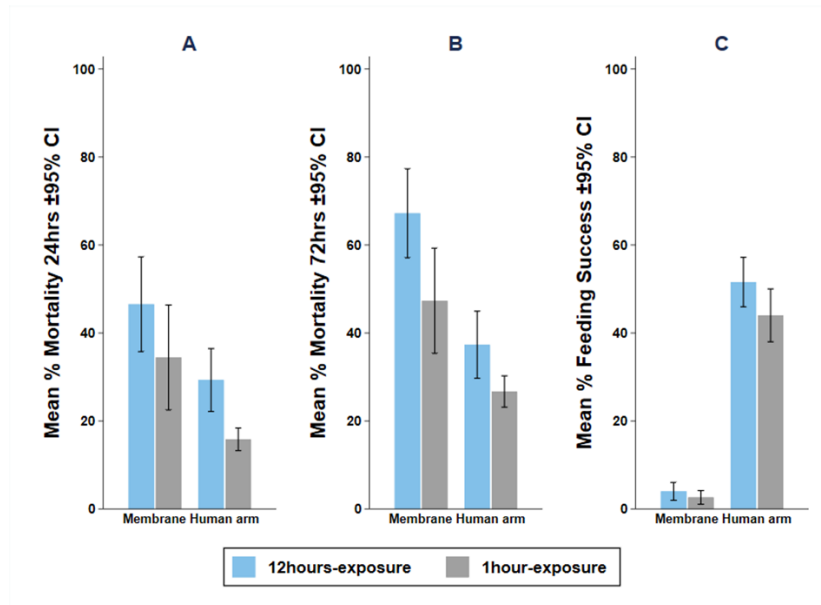
Figure 5: Mean and 95% Confidence Interval of mortality and blood feeding success of resistant *anopheles arabiensis*

Figure shows (A) 24-hours mortality, (B) 72-hours mortality and (C) Blood feeding success with 100 pyrethroid resistant Anopheles *arabiensis* mosquitoes with 12-hours or 1-hour exposure time in the WHO tunnel bioassay using Hemotek® membrane or human arm as bait.

4.1.4 Effects of mosquito density on tunnel test endpoints

Mortality and blood feeding success were not statistically different when either 50 or 100 mosquitoes were used in the tunnel bioassay with rabbit bait for either the susceptible or resistant strains (Table 4). This was consistent for both Interceptor® and Interceptor®G2, unwashed and washed 20 times (Fig.6). No systematic difference in agreement between methods was observed by Bland and Altman methods (Appendix 6). The mean difference was -4.54% (-31.62-22.54) in blood feeding success and 1.71% (-28.71-32.12) in mortality at 72hrs. Furthermore, when tested using the pyrethroid resistant strain the 50-rabbit bioassay predicted the superiority of Interceptor®G2 to Interceptor® similar to the 100-rabbit (Table 5). It was observed that the odds of blood feeding success were higher and the odds of mortality were lower with the 50-mosquito density, although this was not significantly different in either case (Table 4).

The difference in the odds of mosquito mortality at 24 hours (M24) or 72-hours (M72) and blood feeding success (BFS) for resistant *Anopheles arabiensis* exposed to Interceptor® G2 or susceptible *Anopheles gambiae* to Interceptor® in the gold standard rabbit-100 and 50-rabbit mosquitoes*.

Table 4: Effects of mosquito density on mortality and blood feeding

Species/Density	BFS			M24			M72		
	% (95% CI)	OR (95%CI)	P-value	% (95% CI)	OR (95%CI)	P-value	% (95% CI)	OR (95%CI)	P-value
Susceptible *An. gambiae* with Interceptor®									
100 Mosquitoes	5.8 (3.4-8.2)	1		98.3 (97.5 - 99.1)	1		99.1 (98.6 - 99.6)	1	
50 Mosquitoes	9.1 (6.6-11.6)	2.35 (0.80-6.92)	0.122	98.4 (97.5 - 99.3)	1.10 (0.32 - 3.72)	0.882	99.6 (99.3 - 99.9)	1.80 (0.43-7.54)	0.421
Resistant *An. arabiensis* with Interceptor®G2									
100 Mosquitoes	12.5 (8.9-16.0)	1		51.8 (41.9 - 61.7)	1		73.9 (66.7 - 81.2)	1	
50 Mosquitoes	18.3 (13.3-23.2)	1.54 (0.74-3.22)	0.249	45.1 (40.7 - 49.6)	0.69 (0.23-2.12)	0.518	70.0 (67.0 - 73.1)	0.65 (0.25-1.67)	0.375

* Mosquitoes were exposed for 12 hours in the tunnel. Data presented are mean proportion (%) with 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% confidence interval (95% CI) adjusted for net type and condition.
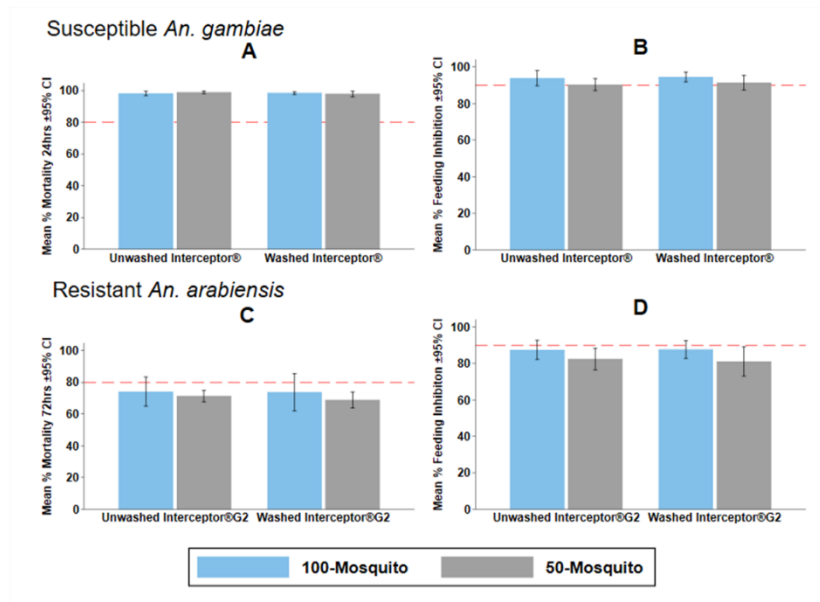
Figure 6: Mean and Confidence Interval of mortality and feeding for susceptible and resistance Anopheles mosquito

Figure shows (A) 24-hours mortality, (B) Blood feeding inhibition of Interceptor® ITN with 100 vs 50 pyrethroid susceptible Anopheles *gambiae*; (C) 72-hours mortality, (D) Blood feeding inhibition of Interceptor®G2 ITN with 100 vs 50 pyrethroid resistant *Anopheles arabiensis* in the WHO tunnel test. Red dashed line depicts WHO minimum bio-efficacy thresholds of ≥ 80% mortality and ≥ 95% blood feeding inhibition.

However, when considering the superiority of Interceptor® and Interceptor® G2 the lower mosquito density (50) resulted in higher blood feeding success in the Interceptor® G2 arm (Table 5). This indicates that mosquitoes at high density are either interacting with each other to disturb each other from feeding, or discomfort from high biting rates is making the host more defensive. This increased blood feeding success is likely translating into the lower odds of mortality observed for washed Interceptor® G2 relative to Interceptor® using 50 mosquitoes (OR: 1.07, 95%CI: 0.85-1.34, p=0.579) compared to 100 mosquitoes (OR: 1.31, 95%CI: 1.12-1.54, p=0.001) (Table 5). This observation underlines the importance of consistent control blood feeding success on mortality estimates from the WHO tunnel test and should always be recorded and reported.

The difference in the odds of mosquito mortality at 24 hours (M24) or 72-hours (M72) and blood feeding success (BFS) for pyrethroid resistant *Anopheles arabiensis* exposed to Interceptor® G2 and Interceptor® in the gold standard rabbit-100 and 50-rabbit mosquitoes*.

Table 5: Superiority of Interceptor®G2 over Interceptor® using 100 versus 50 resistant mosquitoes

| Treatment | 100-Rabbit | | | | 50-Rabbit | | | |
| | BFS | | M72 | | BFS | | M72 | |
	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value	OR (95%CI)	P-value
Overall								
Interceptor®	1		1		1		1	1
Interceptor®G2	1.76 (1.55-1.99)	< 0.001	1.23 (1.13-1.33)	< 0.001	12.93 (9.63-17.36)	< 0.001	1.41 (1.26-1.57)	< 0.001
Unwashed								
Interceptor®	1		1		1		1	1
Interceptor®G2	1.64 (1.38-1.95)	< 0.001	1.15 (1.02-1.29)	0.018	8.50 (5.95-12.15)	< 0.001	1.83 (1.56-2.14)	< 0.001
Washed 20x								
Interceptor®	1		1		1		1	1
Interceptor®G2	1.90 (1.58-2.27)	< 0.001	1.31 (1.17-1.47)	< 0.001	24.34 (14.16-41.85)	< 0.001	1.07 (0.85-1.34)	0.432

* Mosquitoes were exposed for 12 hours in the tunnel. Data presented are mean proportion (%) with a 95% confidence interval (95% CI) as well as odds ratios (OR) derived from regression analysis with a 95% confidence interval (95% CI) adjusted for net conditions.

4.1.5 Possibility to replace standard bait with the membrane feeding

The membrane assay with 50 mosquitoes (membrane-50) did not measure statistically different 24-hour mortality or 72-hour mortality compared to the rabbit with 100 mosquitoes (rabbit-100) (Table 6) when testing pyrethroid only Interceptor® or Interceptor®G2 against pyrethroid resistant *An. arabiensis*. Again, blood feeding success was different, with far higher success in the rabbit-100 assay.

The difference in the odds of mosquito mortality at 72-hours (M72) and blood feeding success (BFS) for resistant *An .arabiensis* was measured between the gold standard rabbit assay with 100 mosquitoes and the membrane assay with 50 mosquitoes.

Table 6: Comparison of the membrane assay to the gold standard with rabbit assay

Treatment/Assay	BFS % (95%CI)	BFS OR (95%CI)	BFS P-value	M24 % (95%CI)	M24 OR (95%CI)	M24 P-value	M72 % (95%CI)	M72 OR (95%CI)	M72 P-value
Interceptor®									
100 Rabbit	7.9 (4.1-11.8)	1		56.4 (45.3-67.6)			169.4 (60.0-78.8)	1	
50 Membrane	1.2 (0.8-1.7)	0.19 (0.08-0.45)	< 0.001	52.5 (45.6-59.4)	0.39 (0.10-1.61)	0.195	73.0 (66.9-79.0)	0.54 (0.14-2.06)	0.370
Interceptor®G2									
100 Rabbit	12.5 (9.0-16.0)	1		51.8 (42.0-61.7)			173.9 (66.7-81.1)	1	
50 Membrane	2.3 (1.3-3.2)	0.17 (0.09-0.30)	< 0.001	56.4 (49.8-63.1)	1.10 (0.51-2.36)	0.814	83.0 (79.1-86.9)	1.50 (0.75-2.98)	0.251

* Mosquitoes exposed for 12 hours in the tunnel. Data presented are mean proportion (%) with a 95% confidence interval (95% CI) as well as odds ratios (OR) derived from regression analysis with a 95% confidence interval (95% CI) adjusted for net type.

However, when used for predicting the difference in bio-efficacy between Interceptor® and Interceptor® G2 both assays measured in the same way (Fig.7) and both predicted superior odds of 72-hour mortality for Interceptor® G2 (100-rabbit OR: 1.23 (1.10-1.38), $p<0.0001$; 50-membrane 1.79 (1.50-2.14) $p<0.0001$) and inferior reduction in blood feeding (100-rabbit OR: 1.76 (1.47-2.10), $p<0.0001$; 50-membrane 1.87 (1.05-3.33) $p=0.033$) with Interceptor® G2 as compared to Interceptor® (Table 7). No systematic difference was observed in agreement for membrane-50 and rabbit-100 by Bland and Altman methods with a mean difference of 9.06 % (-11.42-29.54) on blood feeding and -5.43 % (-50.3-39.45) on mortality (Appendix 7).
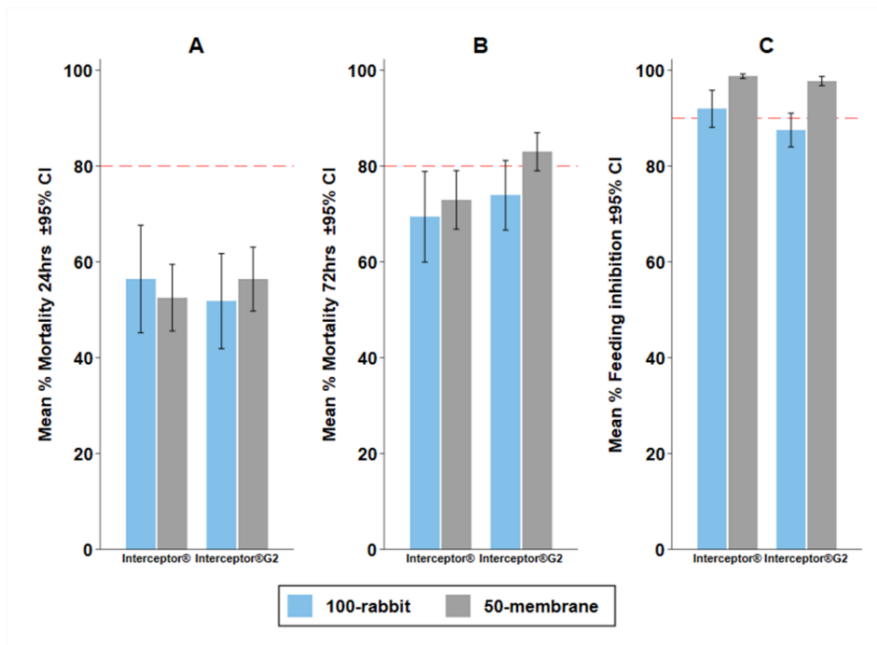
Figure 6: Mean and Confidence Interval of mortality and feeding for susceptible and resistance Anopheles mosquito

Figure shows(A) 24-hour mortality, (B) 72-hour mortality and (C) Blood feeding inhibition for Interceptor® and Interceptor®G2 nets against pyrethroid resistant *Anopheles arabiensis* with 100-rabbit (rabbit bait and density of 100 mosquitoes) and 50-membrane (Hemotek® membrane bait and density of 50 mosquitoes) in the WHO tunnel test. Red dashed line depicts WHO minimum bio-efficacy thresholds of ≥ 80% mortality and ≥ 95% blood feeding inhibition.

The difference in the odds of mosquito mortality at 72-hours (M72) and blood feeding success (BFS) for resistant *Anopheles arabiensis* measuring the superiority of Interceptor®G2 and Interceptor® with the gold standard with 100-rabbit compared to 50-membrane bioassays.

Table 7: Superiority of Interceptor®G2 over Interceptor® comparing membrane assay to the gold standard assay

Treatment	100-Rabbit				50-Membrane			
	BFS		M72		BFS		M72	
	OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
Overall								
Interceptor®		1		1		1		1
Interceptor®G2	1.76 (1.47-2.10)	< 0.001	1.23 (1.10-1.38)	< 0.001	1.87 (1.05-3.33)	0.033	1.79 (1.50-2.14)	< 0.001
Unwashed								
Interceptor®		1		1		1		1
Interceptor®G2	1.64 (1.28-2.09)	< 0.001	1.15 (0.98-1.35)	0.094	2.34 (1.11-4.93)	0.025	1.81 (1.43-2.29)	< 0.001
Washed								
Interceptor®		1		1		1		1
Interceptor®G2	1.90 (1.47-2.45)	< 0.001	1.31 (1.12-1.54)	0.001	1.26 (0.49-3.20)	0.634	1.82 (1.39-2.37)	< 0.001

*For the gold standard, 100-mosquitoes with rabbit and 50-mosquito with 2 Hemotek® membrane feeders augmented with worn socks were used in the WHO tunnel bioassay, adjusted for net type and condition.

4.2 Discussion

Based on the current work it is proposed that a larger number of nets or two samples per ITN can be tested using 50 mosquitoes per replicate to improve laboratory throughput. Biological durability monitoring requires large sample sizes as nets are exposed to highly variable use patterns (Abilio *et al.*, 2020; Kilian *et al.*, 2015; Mutuku *et al.*, 2013) and environmental conditions (Allan, 2012; Kilian *et al.*, 2021) that result in a high degree of heterogeneity between individual nets. The goal of biological durability monitoring is the precise estimation of the biological efficacy of a population of ITNs. As the ITN is the unit of replication, greater precision is obtained by evaluating larger numbers of ITN samples.

The current experiment confirmed that using the Hemotek® membrane feeding system as a blood source together with worn sock emitting human odor with a replicate size of 50 mosquitoes estimates similar mortality and feeding inhibition as the standard WHO tunnel bioassay with rabbit and a replicate size of 100 mosquitoes for both pyrethroid and mixture pyrethroid and chlorfenapyr ITNs (WHOPES, 2013). This results suggest membrane bioassay can evaluate the difference between ITNs because the membrane assay estimates the superiority of Interceptor®G2 over Interceptor® on the M72 outcome using metabolic resistant mosquitoes. Similar results was observed when superiority of same nets measured by the gold standard rabbit-100 assay and has been consistently seen in other studies in the WHO tunnel, I-ACT, and experimental hut (Kibondo *et al.*, 2022). It was also able to predict superior blood feeding inhibition of Interceptor® which has a higher concentration of the pyrethroid alpha-cypermethrin (200 mg/m^2 alpha-cypermethrin in Interceptor® and 100 mg/m^2 alpha-cypermethrin in Interceptor® G2). Being able to test differences between products is one of the parameter in durability monitoring bioassays that track the bio-efficacy of ITNs over time (biological durability) and compare them to unwashed positive controls (Lissenden *et al.*, 2022).

Having a reliable bioassay that can be conducted routinely without animal welfare concerns will be extremely useful. The data generated by the current work are promising and further work is needed to improve mosquito feeding success on the membrane as it was seen that differences in blood feeding success do impact the mortality estimates. While this did not impact predictions of superiority and therefore mortality can still be compared to an unwashed positive control net, if

thresholds are used i.e. the proportion of nets that meet WHO bio-efficacy criteria, then this might affect the interpretation of the bioassay results. It is recommended that the results are replicated in different laboratories using different mosquito species to optimize the assay. Data from the experiments demonstrated that several factors influenced the mean mortality and feeding inhibition estimated in WHO tunnel tests (Lorenz *et al.*, 2020; Mansiangi *et al.*, 2020; Oxborough *et al.*, 2015).

4.2.1 Impact of the bait

The use of different baits had an enormous influence on mosquito feeding success. Using a human arm as bait, feeding inhibition is substantially lower compared to a membrane or rabbit baits (Hossain, 1989). This has also been seen in early versions of the tunnel test (Hossain, 1989). This preference for the human arm is unsurprising since the colony used in the experiments is anthropophilic. Therefore, although it is more representative of end-user conditions, the use of a human is not recommended for ITN evaluation (WHOPES, 2013) because the results were not comparable to those of the rabbit bioassay that has been shown to predict the results of experimental hut trials in this setting (Kibondo, 2022) and elsewhere (N'Guessan *et al.*, 2016; Oxborough *et al.*, 2015; R, 2007). Study findings using *An. arabiensis* mosquitoes were consistent with existing literature on vector host preference (Kweka, 2010; Mukabana *et al.*, 2002; Takken & Verhulst, 2013) confirming that mosquitoes are most attracted by humans as bait, followed by rabbits, and were least attracted to the membrane. Lower attraction in assays using the Hemotek® membrane system and rabbits reduces the number of mosquitoes passing the ITN tested, resulting in higher feeding inhibition compared to the human arm as bait. Several other studies have shown that host-seeking *An. arabiensis* are more attracted to humans than live animals (Pates *et al.*, 2001; Takken & Knols, 1999). The lower attraction and consequent higher feeding inhibition when using a membrane are likely due to the absence of carbon dioxide (CO_2) and less heat produced that increases mosquito responses to kairomones (van Loon *et al.*, 2015) and the small size of the membrane feeder's surface which reduces the amount of heat and moisture available, which are both important short-range attractants to mosquitoes (Hawkes *et al.*, 2017; Kellogg, 1970; Khan & Maibach, 1971). The validity of the experiment relies on the negative control feeding success of (>50%) for rabbits. In this assay with the membrane, augmentation with socks that contained human kairomones improved the attraction of the membrane to mosquitoes (Okumu *et al.*, 2010).

However, it was not possible to use the same threshold value for feeding success with the less attractive membrane. For this reason, further work is needed to optimize the attraction of the membranes for use in the WHO tunnel test. Further improvements to the attractiveness of the membrane could be achieved by making a larger surface area available (Kweka, 2010; Romano *et al.*, 2018) and the addition of 2-butanone (Mburu *et al.*, 2017) or CO_2 (Morimoto *et al.*, 2021) to augment mosquito response to kairomones until 50% feeding success in the negative control is consistently achieved.

4.2.2 Impact of exposure time

Exposure time was important with a 12-hours exposure increasing both mortality and feeding success indicating that the mosquitoes make repeated contact with the ITN sample overnight. Consistently, prolonged exposure (12 hrs) increased the efficacy of insecticide and host-seeking activities compared to 1hr, resulting in increased mortality as a consequence of a higher dose of insecticide picked up by the mosquitoes when resting, bouncing, and passing the ITNs repeatedly. This is also likely in experimental huts and in the community where ITNs are in use. Therefore, the use of a 12-hour overnight exposure is recommended. For insecticides that require the mosquitoes to be metabolically active, such as chlorfenapyr, prolonging exposure to 12 hours allows the conversion of parent molecules into active forms, as a consequence of mosquitoes' metabolic activity when flying in the tunnel. Interestingly, results show that with either the pyrethroid only Interceptor® or the pyrethroid-chlorfenapyr Interceptor®G2 ITNs higher mortality was observed among unfed mosquitoes. Therefore, the results of this study underline the WHO recommendation that feeding success should always be reported when conducting WHO tunnel tests as low feeding rates will affect the interpretation of results.

4.2.3 Effects of mosquito density

It was observed that the use of 50 or 100 mosquitoes per testing sample with the rabbit bait did not significantly alter the mortality and blood feeding success measured with either resistant *An. arabiensis* or susceptible *An. gambiae* for the pyrethoid only net or the mixture ITNs. These results suggest that fewer mosquitoes can be used in WHO tunnel bioassays and still correctly measure the efficacy of ITNs. As would be expected, with 50 mosquitoes there is a slight increase in blood feeding success and a consequent slight decrease in mortality compared to assays using 100

mosquitoes. Higher feeding success at lower density is likely due to less competition between mosquitoes on the membrane during host-seeking (Timinao *et al.*, 2021), which may also reduce the host defensiveness of the rabbit (Anderson & Brust, 1996; Edward, 1986). Increasing the number of mosquitoes in the tunnel may lead to density-dependent mortality effects of crowding as mosquitoes can collide each other when at high density (Styer *et al.*, 2007). Results suggest that regardless of insecticides on ITNs tested from BASF brand, mortality was higher among unfed mosquitoes, revealing an impact of blood feeding on increased mosquito resilience to insecticides. A similar study on the effects of bites through permethrin nets shows successfully fed mosquitoes survive longer than unfed ones (Hauser *et al.*, 2019). This reported for chlorfenapyr, where the efficacy of chlorfenapyr showed mortality was lower among blood-fed mosquitoes compared to unfed (Oxborough *et al.*, 2013). Blood feeding elevates detoxifying enzymes (glutathione, monooxygenase), which then assist in the detoxification of insecticides (Machani *et al.*, 2019), although this did not translate into lower bio-efficacy with Interceptor® G2 as upregulation of metabolization converts the parent molecule into the potent n-dealkylated form that elicits increased mosquito mortality(Michael, 2021). It is also important to report control blood feeding success because unfit colony mosquitoes are less likely to fly and feed, which reduces the likelihood that the mosquitoes contact treated nets (Hauser *et al.*, 2019).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Here, it was demonstrated that using 50 or 100 mosquitoes with the rabbit gives similar results with no systematic bias for both pyrethroid and pyrethroid-chlorfenapyr ITNs. The lower density can be used for the WHO tunnel test when testing pyrethroid Interceptor® and pyrethroid-chlorfenapyr Interceptor®G2. Reducing the number of mosquitoes per test decreases its cost and allows a larger number of net samples to be tested at a time. Larger sample sizes will give greater precision when estimating ITN efficacy since the unit of replication in ITNs testing is the bioassay (cone, tunnel, I-ACT, Hut) and not the mosquito within that assay. Furthermore, we provide the first evidence that membrane feeding systems can be used as an alternative to rabbit bait in WHO tunnel assays. Membrane assay shows a promising comparison to the gold standard WHO tunnel test on both the mortality and feeding success endpoint for the ITNs although control-feeding success is lower due to the lower attraction of the membrane to host-seeking mosquitoes. Using membrane feeding systems instead of rabbits or other animals in WHO tunnel assays highlight the future solution regarding existing ethical issue concerning animal welfare and makes the tests simpler to perform.

5.2 Recommendations

Findings from this study light the opportunity of optimize membrane assay and use of fewer mosquitoes when evaluating ITNs bio-efficacy and we recommend the following:

(i) Improve the feeding success of the Hemotek® membrane feeding system as a replacement for rabbits in the WHO tunnel test is essential.

(ii) Corroborate our findings by validation of the membrane assay in additional product testing facilities to demonstrate its reproducibility in different setting.

(iii) NIMR through its entomology center may adopting the modified WHO tunnel bioassay for supporting NMCP in conducting quality assurance of incoming and old ITNs.

REFERENCES

Abílio, A. P., Obi, E., Koenker, H., Babalola, S., Saifodine, A., Zulliger, R., Swamidoss, I., Ponce de Leon, G., Alfai, E., Blaufuss, S., Olapeju, B., Harig, H., & Kilian, A. (2020). Monitoring the durability of the long-lasting insecticidal nets MAGNet and Royal Sentry in three ecological zones of Mozambique. *Malaria Journal,* 19(1), 209.

Ouédraogo, A.L., Guelbéogo, W.M., Cohuet, A., Morlais, I., King, J.G., Gonçalves, B.P.,Bastiaens, G.J., Thiombiano, F., Sattabongkot, J., Wu, Y., Coulibaly, M.B., Ibrahima, B., Jones,S., Drakeley, C.J., Dinglasan, R.R., & Bousema, T. (2013). A protocol for membrane feeding assays to determine the infectiousness of P. falciparum naturally infected individuals to *Anopheles gambiae*. *Malaria World Journal*, 12(1), 42-47.

Allan, R., O'Reilly, L., Gilbos, V., & Kilian, A.(2012). An Observational Study of Material Durability of Three World Health Organization-Recommended Long-Lasting Insecticidal Nets in Eastern Chad. *AmericanJournalof Tropical Medicine and Hygiene*, 87(3), 407-411.

Anderson, R. A., & Brust, R. A. (1996). Blood Feeding Success of *Aedes Aegypti and Culex Nigripalpus* (*Diptera :Cullicidae*) in Relation to Defensive Behavior of Japanese Quail (Coturnix Japonica) in the Laboratory. *Journal of Vector Ecology*, 21(1), 94-104.

Awono-Ambene, H. P., Diawara, L., & Robert, V. (2001). Comparison of direct and membrane feeding methods to infect *Anopheles arabiensis* with *Plasmodium falciparum. The American Journal of Tropical Medicine and Hygiene*, *64*(1-2), 32–34. https://doi.org/10.4269/ajtmh.2001.64.32.

Birget, P. L., & Koella, J. C. (2015). An Epidemiological Model of the Effects of Insecticide-Treated Bed Nets on Malaria Transmission. *PloS One*, *10*(12), e0144173.

Bland, J. M., & Altman, D. G. (1999). Measuring agreement in method comparison studies. *Statistical Methods in Medical Research*, *8*(2), 135–160. https://doi.org/10.1177/096228029900800204.

Carnevale, P., Bitsindou, P., Diomandé, L., & Robert, V. (1992). Insecticide impregnation can restore the efficiency of torn bed nets and reduce man-vector contact in malaria endemic areas. *Transactions of the Royal Society of Tropical Medicine*, 86(4),362-364.

Chandre, F., Darriet, F., Duchon, S., Finot, L., Manguin, S., Carnevale, P., & Guillet, P. (2000). Modifications of pyrethroid effects associated with *kdr* mutation in *Anopheles gambiae*. *Medical and Veterinary Entomology*, *14*(1), 81–88. https://doi.org/10.1046/j.1365-2915.2000.00212.

Cook, J., Tomlinson, S., Kleinschmidt, I., James, D. M., Akogbeto, M., Adechoubou, A., Massougbodji, A., Okê-Sopoh, M., Corbel, V., Cornelie, S., Hounto, A., Etang, J., Awono-Ambene, H. P.,Bigoga, J., Mandeng, S. E., Njeambosay, B., Tabue, R., Kouambeng, C., Fondjo, E.,… Nkuni, Z. J. (2018). Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: Trends in pyrethroid resistance during a WHO-coordinated multi-country prospective study. *Parasiteand Vectors,* 11(1), 550.

Damiens, D., Soliban, S.M., Balestrino, F., Alsir, R., Vreysen, M.J., & Gilles, J.R.,. (2013). Different Blood and Sugar Feeding Regimes Affect the Productivity of *Anopheles arabiensis* Colonies *(Diptera: Culicidae). Journal of Medical Entomology*, 50(2), 336-343.

David, M. D. (2021). The potential of pro-insecticides for resistance management. *Pest Management Science*, 77(8), 3631-3636.https://doi.org/10.1002/ps.6369.

Debboun, M., & Wagman, J. (2004). In vitro repellency of N,N-diethyl-3-methylbenzamide and N,N-diethylphenylacetamide analogs against *Aedes aegypti and Anopheles stephensi* (Diptera: Culicidae). *Journal of Medical Entomology*, *41*(3), 430–434.

Dekker T., Takken W., & Cardé. (2001). Structure of host-odour plumes influences catch of *Anopheles gambiae s.s. and Aedes aegypti* in a dual-choice olfactometer. *Physiological Entomology*. 2001 Jun;26(2):124-134.https://doi.org/10.1046/j.1365-3032.2001.00225.

Diallo, M., Touré, A. M., Traoré, S. F., Niaré, O., Kassambara, L., Konaré, A., Coulibaly, M., Bagayogo, M., Beier, J. C., Sakai, R. K., Touré, Y. T., & Doumbo, O. K. (2008). Evaluation and optimization of membrane feeding compared to direct feeding as an assay for infectivity. *Malaria Journal*, *7*, 248. https://doi.org/10.1186/1475-2875-7-248.

Edman,Webber, & Kale. (1972). Effect of mosquito density on the interrelationship of host behavior and mosquito feeding success. *American Journal of Tropical Medicine and Hygiene*, 21(4), 487-491.

Edward, D. W., Edman, J. D. (1986). Influence of defensive behavior of eastern chipmunks and gray squirrels (Rodentia: Sciuridae) on feeding success of *Aedes triseriatus* (Diptera: Culicidae). *Journal of Medical Entomology*, 23(1), 1-10.

Gleave, K., Lissenden, N., Chaplin, M., Choi, L.,& Ranson, H.. (2021). Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Journal Cochrane Database of Systematic Reviews*(5).

Hauser, G., Thiévent, K., & Koella, J. C. (2019). The ability of *Anopheles gambiae* mosquitoes to bite through a permethrin-treated net and the consequences for their fitness. *Scientific reports*, 9(1), 8141. https://doi.org/10.1038/s41598-019-44679-1.

Hawkes, F. M., Dabiré, R. K., Sawadogo, S. P., Torr, S. J., & Gibson, G. (2017). Exploiting Anopheles responses to thermal, odour and visual stimuli to improve surveillance and control of malaria. *Scientific reports*, 7(1), 1-9.

Hawley, W. A., Phillips-Howard, P. A., Ter Kuile, F . O., Terlouw, D. J., Vulule, J. M., Ombok, M., Nahlen, B. L., Gimnig, J. E., Kariuki, S. K., Kolczak, M. S., & Hightower, A. W. (2003). Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western kenya [Journal]. *American Journal of Tropical Medicine and Hygiene*, 68(4_suppl), 121-127.https://doi.org/10.4269/ajtmh.2003.68.121.

Hossain, I. M.,&Curtis, F. C.,. (1989). Permethrin-impregnated bednets: behavioural and killing effects on mosquitoes [Article]. *Medical and Veterinary Entomology*, 3, 1-10.

Hougard, J. M., Duchon, S., Darriet, F., Zaim, M., Rogier, C., & Guillet, P. (2003). Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. *Bulletin of the World Health Organization*, *81*(5), 324–333.

Johnson, P. C., Barry, S. J., Ferguson, H. M., & Müller, P. (2015). Power analysis for generalized linear mixed models in ecology and evolution. *Methods in Ecology and Evolution.*

Kaufman, A. S. (2011). Methods in Anopheles Research Manual [Report]. Book, 419.

Kellogg, F. E. (1970). Water vapour and carbon dioxide receptors in *Aedes aegypti. Journal of Insect Physiology,* 16(1), 99-108.

Khan, A. A., & Maibach, H. I. (1971). A study of the probing response of *Aedes aegypti*. *Journal of Economic Entomology,* 64(2), 442-443. https://doi.org/10.1093/jee/64.2.442.

Kibondo, U. A., Odufuwa, O. G., Ngonyani, S. H., Mpelepele, A. B., Matanilla, I., Ngonyani, H., & Moore, S. J. (2022). Influence of testing modality on bioefficacy for the evaluation of Interceptor® G2 mosquito nets to combat malaria mosquitoes in Tanzania. *Parasites & Vectors,* 15(1), 1-18. https://doi.org/10.1186/s13071-022-05207-9.

Kilian, A., Koenker, H., Obi, E., Selby, R. A., Fotheringham, M., & Lynch, M. (2015). Field durability of the same type of long-lasting insecticidal net varies between regions in Nigeria due to differences in household behaviour and living conditions. *Malaria Journal,* 14(1), 1-16.

Kilian, A., Obi, E., Mansiangi, P., Abílio, A. P., Haji, K. A., Blaufuss, S., & Koenker, H. (2021). Variation of physical durability between LLIN products and net use environments: summary of findings from four African countries. *Malaria Journal,* 20(1),1-11.

Kleinschmidt, I., Bradley, J., Knox, T. B., Mnzava, A. P., Kafy, H. T., Mbogo, C., & Donnelly, M. J. (2018). Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: A WHO-coordinated, prospective, international, observational cohort study. *The Lancet Infectious Diseases*, 18(6), 640-649. https:doi.org/10.1016/S1473-3099(18)30172-5.

Koella, J. C., Lynch, P. A., Thomas, M. B., & Read, A. F. (2009). Towards evolution-proof malaria control with insecticides.*Evolutionary Applications*,2(4),469-480.https://doi.org/10.1111/j .1752-4571.2009.00072.

Kweka, E. J., Mwang'onde, B. J., Lyaruu, L., Tenu, F., & Mahande, A. M. (2010). Effect of different hosts on feeding patterns and mortality of mosquitoes (Diptera: Culicidae) and their implications on parasite transmission. *Journal of Global Infectious Diseases*,2(2),121.

Le Menach, A., Takala, S., McKenzie, F. E., Perisse, A., Harris, A., Flahault, A., & Smith, D. L. (2007). An elaborated feeding cycle model for reductions in vectorial capacity of night-biting mosquitoes by insecticide-treated nets. *Malaria Journal*,6(1),1-12.https://doi.org/ 10.1186/ 1475-2875-6-10

Lines, J. D., Myamba, J., & Curtis, C. F. (1987). Experimental hut trials of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania. *Medical and veterinary Entomology, 1(1), 37-51.*

Lissenden, N., Armistead, J. S., Gleave, K., Irish, S. R., Martin, J. L., Messenger, L. A., & Lees, R. S. (2021). Developing consensus standard operating procedures (SOPs) to evaluate new types of insecticide-treated nets. Insects, 13(1), 7. https://www.mdpi.com/2075-4450/13/1/7.

Lorenz, L. M., Bradley, J., Yukich, J., Massue, D. J., Mageni Mboma, Z., Pigeon, O., & Moore, S. J. (2020). Comparative functional survival and equivalent annual cost of 3 long-lasting insecticidal net (LLIN) products in Tanzania: A randomised trial with 3-year follow up. *PLoS Medicine*, 17(9), e1003248.https://doi.org/10.1371/journal.pmed.1003248.

Lorenz, L. M., Keane, A., Moore, J. D., Munk, C. J., Seeholzer, L., Mseka, A., & Moore, S. J. (2013). Taxis assays measure directional movement of mosquitoes to olfactory cues. *Parasites & vectors*, 6(1), 1-12. https://doi.org/10.1186/1756-3305-6-131.

Machani, M. G., Ochomo, E., Sang, D., Bonizzoni, M., Zhou, G., Githeko, A. K., & Afrane, Y. A. (2019). Influence of blood meal and age of mosquitoes on susceptibility to pyrethroids in *Anopheles gambiae* from Western Kenya. *Malaria Journal*, 18(1), 1-9.

Magesa, S. M., Wilkes, T. J., Mnzava, A. E. P., Njunwa, K. J., Myamba, J., Kivuyo, M. D. P., & Curtis, C. F. (1991). Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria Part 2. Effects on the malaria vector population. *Acta tropica*, 49(2), 97-108.

Malima, R. C., Oxborough, R. M., Tungu, P. K., Maxwell, C., Lyimo, I., Mwingira, V., & Rowland, M. W. (2009).Behavioural and insecticidal effects of organophosphate-,carbamate-and pyrethroid-treated mosquito nets against African malaria vectors.*Medical and Veterinary Entomology*, 23(4), 317-325.

Malima, R. C., Magesa, S. M., Tungu, P. K., Mwingira, V., Magogo, F. S., Sudi, W., & Rowland, M. (2008). An experimental hut evaluation of Olyset® nets against anopheline mosquitoes after seven years use in Tanzanian villages. *Malaria Journal*, 7(1), 1-8.Https://doi.org/10.1186/1475-2875-7-38.

Mansiangi, P., Umesumbu, S., Etewa, I., Zandibeni, J., Bafwa, N., Blaufuss, S., & Kilian, A. (2020). Comparing the durability of the long-lasting insecticidal nets DawaPlus® 2.0 and DuraNet© in northwest Democratic Republic of Congo.*Malaria Journal*,19(1),1-17.

Massue, D. J., Lorenz, L. M., Moore, J. D., Ntabaliba, W. S., Ackerman, S., Mboma, Z. M., & Moore, S. J. (2019). Comparing the new Ifakara Ambient Chamber Test with WHO cone and tunnel tests for bioefficacy and non-inferiority testing of insecticide-treated nets. *Malaria Journal*, 18(1), 1-15.https://doi.org/10.1186/s12936-019-2741.

Michael D. David . (2021). The potential of pro-insecticides for resistance management.*Journal of Pest Management Science,*77(8),36313636.https://onlinelibrary.wiley.com/doi/10.1002/ps.6369.

Mburu, M. M., Mweresa, C. K., Omusula, P., Hiscox, A., Takken, W., & Mukabana, W. R. (2017). 2-Butanone as a carbon dioxide mimic in attractant blends for the Afrotropical malaria mosquitoes *Anopheles gambiae and Anopheles funestus*. *Malaria Journal*, 16(1), 1-9.

Morimoto, Y., Kawada, H., Kuramoto, K. Y., Mitsuhashi, T., Saitoh, T., & Minakawa, N. (2021). New mosquito repellency bioassay for evaluation of repellents and pyrethroids using an attractive blood-feeding device. *Parasites&Vectors*,14(1),1-12.https://doi.org/10.1186/s13071-021-04656.

Mosha, J. F., Kulkarni, M. A., Lukole, E., Matowo, N. S., Pitt, C., Messenger, L. A., & Protopopoff, N. (2022). Effectiveness and cost-effectiveness against malaria of three types of dual-active-ingredient long-lasting insecticidal nets (LLINs) compared with pyrethroid-only LLINs in Tanzania: A four-arm, cluster-randomised trial. *The Lancet*, 399(10331), 1227-1241.

Mosha, F. W., Lyimo, I. N., Oxborough, R. M., Malima, R., Tenu, F., Matowo, J., & Rowland, M. (2008). Experimental hut evaluation of the pyrrole insecticide chlorfenapyr on bed nets for the control of Anopheles arabiensis and Culex quinquefasciatus. *Tropical Medicine & International Health*, 13(5), 644-652./https://doi.org/10.1111/j.1365-3156.2008.02058.

Mukabana, W. R., Takken, W., Coe, R., & Knols, B. G. (2002). Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*. *Malaria Journal*, 1(1), 1-8.https://doi.org/10.1186/1475-2875-1-17.

Mutuku, F. M., Khambira, M., Bisanzio, D., Mungai, P., Mwanzo, I., Muchiri, E. M., & Kitron, U. (2013). Physical condition and maintenance of mosquito bed nets in Kwale County, coastal Kenya. *Malaria Journal*,12(1),1-14.

N'Guessan, R., Ngufor, C., Kudom, A. A., Boko, P., Odjo, A., Malone, D., & Rowland, M. (2014). Mosquito nets treated with a mixture of chlorfenapyr and alphacypermethrin control pyrethroid resistant *Anopheles gambiae and Culex quinquefasciatus* mosquitoes in West Africa. *PloS one,* 9(2), e87710.https://doi.org/10.1371/journal.pone.0087710.

N'Guessan, R., Odjo, A., Ngufor, C., Malone, D., & Rowland, M. (2016). A Chlorfenapyr Mixture Net Interceptor® G2 shows high efficacy and wash durability against resistant mosquitoes in West Africa. *PLoS One*, 11(11), e0165925.https://doi.org/10.1371/journal.pone.0165925.

Gunathilaka, N., Ranathunge, T., Udayanga, L., & Abeyewickreme, W. (2017). Efficacy of blood sources and artificial blood feeding methods in rearing of *Aedes aegypti* (Diptera:Culicidae) for sterile insect technique and incompatible insect technique approaches in Sri Lanka. *Bio Medical Research International*, 2017.https://doi.org/10.1155/2017/3196924.

Okumu, F., Biswaro, L., Mbeleyela, E., Killeen, G. F., Mukabana, R., & Moore, S. J. (2014). Using nylon strips to dispense mosquito attractants for sampling the malaria vector Anopheles gambiae ss. *Journal of Medical Entomology*, 47(2), 274-282.https://doi.org/10.1603/me09114.

Ouédraogo, A.L., Guelbéogo, W.M., Cohuet, A., Morlais, I., King, J.G., Gonçalves, B.P., Bastiaens, G.J., Thiombiano, F., Sattabongkot, J., Wu, Y., Coulibaly, M.B., Ibrahima, B., Jones, S., Drakeley, C.J., Dinglasan, R.R., & Bousema, T. (2013). A protocol for membrane feeding assays to determine the infectiousness of P. falciparum naturally infected individuals to Anopheles gambiae. *Malaria World Journal, 12(1), 42-47.*

Oxborough, R. M., N'Guessan, R., Jones, R., Kitau, J., Ngufor, C., Malone, D., & Rowland, M. W. (2015). The activity of the pyrrole insecticide chlorfenapyr in mosquito bioassay: Towards a more rational testing and screening of non-neurotoxic insecticides for malaria vector control. *Malaria Journal*, 14(1), 1-11.https://doi.org/10.1186/s12936-015-0639.

Oxborough, R. M., Kitau, J., Matowo, J., Feston, E., Mndeme, R., Mosha, F. W., & Rowland, M. W. (2013). ITN mixtures of chlorfenapyr (pyrrole) and alphacypermethrin (pyrethroid) for control of pyrethroid resistant *Anopheles arabiensis and Culex quinquefasciatus. PLoS One*, 8(2), e55781.https://doi.org/10.1371/journal.pone.0055781.

Oxborough, R. M., N'Guessan, R., Jones, R., Kitau, J., Ngufor, C., Malone, D., & Rowland, M. W. (2015). The activity of the pyrrole insecticide chlorfenapyr in mosquito bioassay: Towards a more rational testing and screening of non-neurotoxic insecticides for malaria vector control. *Malaria Journal*, 14(1), 1-11.https://doi.org/10.1186/s12936-015-0639.

Parker, J. E., Angarita, N. C., Gleave, K., Mashauri, F., Abe, M., Martine, J., & McCall, P. J. (2017). Host-seeking activity of a Tanzanian population of *Anopheles arabiensis* at an insecticide treated bed net. *Malaria Journal,* 16(1), 1-14.https://doi.org/10.1186/s12936-017-1909-6.

Pates, H. V., Takken, W., Stuke, K., & Curtis, C. F. (2001). Differential behaviour of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human and cow odours in the laboratory. *Bulletin of entomological research*, 91(4), 289-296.https://doi.org/10.1079/ber200198.

PATH. (2022). New Nets Project interim report 2022. Report, Vol, 1-96.

Phasomkusolsil, S., Tawong, J., Monkanna, N., Pantuwatana, K., Damdangdee, N., Khongtak, W., & Schuster, A. L. (2013). Maintenance of mosquito vectors: Effects of blood source on feeding, survival, fecundity, and egg hatching rates. *Journal of Vector Ecology*, 38(1), 38-45.

Pryce, J., Richardson, M., & Lengeler, C. (2018). Insecticide-treated nets for preventing malaria. *Cochrane Database of Systematic Reviews,* (11). https://doi.org/10.1002/14651858.CD000363.

N'guessan, R., Boko, P., Odjo, A., Akogbeto, M., Yates, A., & Rowland, M. (2007). Chlorfenapyr: A pyrrole insecticide for the control of pyrethroid or DDT resistant Anopheles gambiae *(Diptera: Culicidae)* mosquitoes. *Acta tropica*, 102(1), 69-78.

Raghavendra, K., Barik, T. K., Sharma, P., Bhatt, R. M., Srivastava, H. C., Sreehari, U., & Dash, A. P. (2011). Chlorfenapyr: A new insecticide with novel mode of action can control pyrethroid resistant malaria vectors. *Malaria Journal*, 10(1), 1-7.https://doi.org/10.1186/s12936-015-0836-7.

Robert, V. (1998). Age grading *Anopheles arabiensis*: their gorging and surviving responses using a membrane feeding system. *Parasite,* 5(1), 87-90.https://doi.org/10.1051/parasite/1998051087

Romano, D., Stefanini, C., Canale, A., & Benelli, G. (2018). Artificial blood feeders for mosquitoes and ticks—Where from, where to?. *Acta tropica*, 183, 43-56.

Smallegange, R. C., Verhulst, N. O., & Takken, W. (2011). Sweaty skin: an invitation to bite?. *Trends in Parasitology*, 27(4), 143-148. https://doi.org/10.1016/j.pt.2010.12.009.

Smit, M. R., Ochomo, E. O., Aljayyoussi, G., Kwambai, T. K., Abong'o, B. O., Bousema, T., & Ter Kuile, F. O. (2019). Human direct skin feeding versus membrane feeding to assess the mosquitocidal efficacy of high-dose ivermectin (IVERMAL Trial). *Clinical Infectious Diseases*, 69(7), 1112-1119.

Staedke, S. G., Gonahasa, S., Dorsey, G., Kamya, M. R., Maiteki-Sebuguzi, C., Lynd, Amy., Katureebe, Agaba., Kyohere, M., Mutungi, P., Kigozi, Simon. P., Opigo, J., Hemingway, J., & Donnelly. (2020). Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): A pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *The Lancet*, 395(10232), 1292-1303.

Styer, L. M., Carey, J. R., Wang, J. L., & Scott, T. W. (2007). Mosquitoes do senesce: departure from the paradigm of constant mortality. *The American Journal of Tropical Medicine and Hygiene*, 76(1), 111–117.

Takken, W., & Knols, B. G. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annual Review of Entomology*, 44, 131–157. https://doi.org/10.1146/annurev.ento.44.1.131.

Takken, W., & Verhulst, N. O. (2013). Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*, 58, 433–453. https://doi.org/10.1146/annurev-ento-120811-153618.

Timinao, L., Vinit, R., Katusele, M., Schofield, L., Burkot, T. R., & Karl, S. (2021). Optimization of the feeding rate of *Anopheles farauti s.s.* colony mosquitoes in direct membrane feeding assays. *Parasites & Vectors*, 14(1), 356. https://doi.org/10.1186/s13071-021-04842-y.

Vallejo, A. F., Rubiano, K., Amado, A., Krystosik, A. R., Herrera, S., & Arévalo-Herrera, M. (2016). Optimization of a Membrane Feeding Assay for Plasmodium vivax Infection in *Anopheles albimanus*. *Plos Neglected Tropical Diseases*, 10(6), e0004807.

van Breugel, F., Riffell, J., Fairhall, A., & Dickinson, M. H. (2015). Mosquitoes Use Vision to Associate Odor Plumes with Thermal Targets. *Current Biology*, 25(16), 2123–2129.

Vantaux, A., Lefèvre, T., Dabiré, K. R., & Cohuet, A. (2014). Individual experience affects host choice in malaria vector mosquitoes. *Parasites & vectors*, 7, 249.

WHO. (2017). Report of the Twentieth whopes working group meeting. Report.

WHO. (2021). World Malaria Report 2021. Geneva: World Health Organization; 2021. (Report, Issue.

WHOPES. (2011). Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational. Guideline, 44.

WHOPES. (2013). Guidelines for laboratory and field testing of long lasting insecticidal nets. Guideline, 2 (102).

APPENDICES

Appendix 1: Mean percentage mortality at M24, M72, blood feeding inhibition (BFI) and 95% confidence interval for Interceptor®G2 and Interceptor®

Assay	Blood feeding success	Mortality	
	% BFS (95%CI)	% M24 (95%CI)	% M72 (95%CI)
Control			
12hr-Rabbit	64.8 (51.2 - 78.3)	3.8 (0.8 - 6.8)	7.7 (5.1 - 10.3)
12hr-Membrane	22.8 (10.4 - 35.1)	6.8 (5.9 - 7.6)	8.9 (8.3 - 9.5)
12hr-Human arm	74.4 (67.9 - 80.8)	6.4 (4.9 - 7.8)	11.7 (9.0 - 14.4)
1hr-Membrane	18.1 (12.5 - 23.6)	6.8 (5.0 - 8.7)	12.3 (9.1 - 15.4)
1hr-Human arm	65.5 (50.9 - 80.2)	7.2 (6.4 - 8.0)	9.4 (8.3 - 10.5)
	Blood feeding inhibition	**Mortality**	
Treatment	% BFI (95%CI)	% M24 (95%CI)	% M72 (95%CI)
Interceptor®			
12hr-Rabbit	93.4 (89.0 - 97.8)	58.1 (36.6 - 79.5)	69.9 (50.5 - 89.2)
12hr-Membrane	97.0 (94.3 - 99.7)	49.7 (34.9 - 64.6)	65.6 (52.0 - 79.2)
12hr-Human arm	52.8 (44.3 - 61.2)	35.2 (23.0 - 47.3)	43.0 (29.9 - 56.0)
1hr-Membrane	96.0 (93.3 - 98.7)	24.7 (17.0 - 32.4)	38.9 (26.5 - 51.2)
1hr-Human arm	58.2 (48.7 - 67.7)	20.3 (17.7 - 22.8)	31.1 (26.1 - 36.1)
Interceptor®G2			
12hr-Rabbit	86.5 (81.8 - 91.1)	41.3 (25.5 - 57.1)	62.3 (51.2 - 73.5)
12hr-Membrane	95.4 (92.3 - 98.5)	43.3 (26.3 - 60.3)	68.8 (52.4 - 85.1)
12hr-Human arm	44.1 (37.3 - 50.9)	23.4 (16.5 - 30.4)	31.7 (24.0 - 39.4)
1hr-Membrane	98.8 (97.7 - 99.9)	44.1 (21.4 - 66.8)	55.8 (34.7 - 76.8)
1hr-Human arm	53.8 (45.9 - 61.7)	11.4 (9.1 - 13.6)	22.3 (18.3 - 26.2)

*Mean percentage mortality and 95% confidence interval (95% CI) for the negative control, Interceptor®G2, and Interceptor® at 24 hours post exposure (M24), mortality at 72 hours post exposure (M72), and blood feeding success (BFS) or blood feeding inhibition (BFI).of resistant *Anopheles arabiensis* with 12 hours of exposure time for rabbit, membrane and human arm; and 1 hour exposure time for membrane and human arm in the WHO tunnel test. The negative control thresholds for the WHO tunnel test are blood feeding success $\geq 50\%$ and M24 $\leq 10\%$.

Appendix 2: Mean percentage mortality at M24, M72, blood feeding inhibition (BFI) and 95% confidence interval for Interceptor®G2 and Interceptor® against and Anopheles species.

Assay	Blood feeding success	Mortality	
	% BFS (95%CI)	% M24 (95%CI)	% M72(95%CI)
Control			
100-Mosquito	83.0 (73.6 - 92.4)	4.7 (3.1 - 6.3)	7.8 (6.2 - 9.4)
50-Mosquito	68.3 (56.3 - 80.3)	8.1 (3.5 - 12.8)	10.2 (7.2 - 13.1)
Treatment	Blood feeding inhibition	Mortality	
	% BFI (95%CI)	% M24 (95%CI)	% M72(95%CI)
Susceptible *An. gambiae* with Interceptor®			
Overall			
100- Mosquito	94.2 (91.8 - 96.6)	98.3 (97.5 - 99.1)	99.1 (98.6 - 99.6)
50- Mosquito	90.9 (88.4 - 93.4)	98.4 (97.5 - 99.3)	99.6 (99.3 - 99.9)
Unwashed			
100- Mosquito	93.9 (89.7 - 98.0)	98.2 (96.9 - 99.5)	99.1 (98.2 - 99.9)
50- Mosquito	90.4 (87.1 - 93.6)	98.9 (98.1 - 99.8)	99.7 (99.4 - 100.1)
Washed 20x			
100- Mosquito	94.5 (91.9 - 97.2)	98.4 (97.6 - 99.3)	99.2 (98.6 - 99.7)
50- Mosquito	91.4 (87.5 - 95.4)	97.9 (96.2 - 99.5)	99.5 (99.0 - 99.9)
Resistant *An. arabiensis* with Interceptor®G2			
Overall			
100- Mosquito	87.5 (84.0 - 91.1)	51.8 (41.9 - 61.7)	73.9 (66.7 - 81.2)
50- Mosquito	81.7 (76.8 - 86.7)	45.1 (40.7 - 49.6)	70.0 (67.0 - 73.1)
Unwashed			
100- Mosquito	87.5 (82.2 - 92.7)	54.1 (40.3 - 67.9)	74.1 (65.0 - 83.2)
50- Mosquito	82.4 (76.5 - 88.4)	45.8 (41.4 - 50.2)	71.2 (67.8 - 74.7)
Washed 20x			
100- Mosquito	87.6 (82.7 - 92.5)	49.6 (35.0 - 64.1)	73.8 (62.2 - 85.3)
50- Mosquito	81.1 (73.0 - 89.1)	44.4 (36.5 - 52.3)	68.8 (63.8 - 73.9)

[*] Susceptible *Anopheles gambiae* with Interceptor®, resistant *Anopheles arabiensis* with Interceptor®G2 at 24 hours post exposure (M24), mortality at 72 hours post exposure (M72) and blood feeding success (BFS) or blood feeding inhibition (BFI) with rabbit bait and a density of 50 or 100 mosquitoes in the WHO tunnel test. The negative control thresholds for the WHO tunnel test are blood feeding success $\geq 50\%$ and M24 $\leq 10\%$.

Appendix 3: Mean percentage mortality at M24, M72, blood feeding inhibition (BFI) and 95% confidence interval for Interceptor®G2 and Interceptor® against 50-membrane and 100-rabbit

Assay	Blood feeding success	Mortality	
	% BFS (95%CI)	% M24 (95%CI)	% M72(95%CI)
Control			
100-Rabbit	64.5 (54.8 - 74.2)	3.9(1.8-6.1)	7.9 (6.0-9.7)
50-Membrane	25.9.1 (21.8 - 30.1.2)	8.7 (6.6-10.7)	14.6 (12.8-16.5)
Blood feeding InhibitionMortality			
Treatment	% BFI (95%CI)	% M24 (95%CI)	% M72(95%CI)
Interceptor®			
Overall			
100-Rabbit	98.3 (97.5 - 99.1)	56.4 (45.3 - 67.6)	69.4 (60.0 - 76.6)
50-Membrane	98.8 (98.3 - 99.2)	52.5 (45.6 - 59.4)	73.0 (66.9 - 79.1)
Unwashed			
100-Rabbit	97.6 (96.1 - 99.0)	52.0 (41.8 - 62.2)	65.5 (57.6 - 71.1)
50-Membrane	98.6 (98.0 - 99.3)	45.8 (36.3 - 55.2)	68.8 (58.5 - 79.1)
Washed 20x			
100-Rabbit	87.5 (84.0 - 91.1)	51.8 (41.9 - 61.7)	68.9 (62.7 - 70.2)
50-Membrane	98.9 (98.3 - 99.6)	59.3 (50.2 - 64.4)	73.2 (66.1 - 76.3)
Interceptor®G2			
Overall			
100-Rabbit	81.7 (76.9 - 86.6)	45.1 (40.7 - 49.6)	73.9 (66.7 - 81.1)
50-Membrane	97.7 (96.8 - 98.7)	56.6 (50.0 - 63.1)	82.5 (78.5 - 86.6)
Unwashed			
100-Rabbit	87.5 (82.2 - 92.7)	54.1 (40.3 - 67.9)	74.1 (65.0 - 83.2)
50-Membrane	96.8 (95.2 - 98.4)	52.1 (42.7 - 61.6)	80.1 (73.0 - 87.1)
Washed 20x			
100-Rabbit	87.6 (82.7 - 92.5)	49.6 (35.0 - 64.1)	73.8 (62.2 - 85.3)
50-Membrane	98.7 (97.8 - 99.5)	61.0 (52.2 - 69.8)	85.0 (81.0 - 89.0)

[*]Mean percentage mortality and 95% confidence interval (95% CI) for the negative control, resistant *Anopheles arabiensis* with Interceptor ® or Interceptor®G2 at 24 hours post exposure (M24), mortality at 72 hours post exposure (M72) and blood feeding success (BFS) or blood feeding inhibition (BFI) with rabbit bait and a density of 100 mosquitoes (rabbit-100) or membrane bait and a density of 50 mosquitoes (membrane-50) in the WHO tunnel test. The negative control thresholds for the WHO tunnel test are blood feeding success ≥ 50% and M24 ≤ 10%.
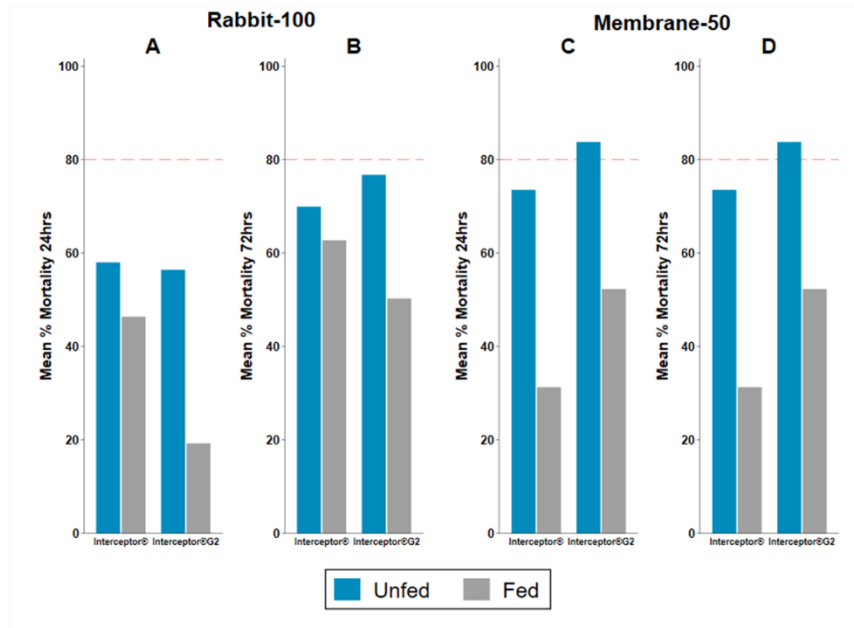
Appendix 4: **Mean percentage mortality at (A) 24-hours and (B) 72-hours with rabbit-100, (C) at 24-hours and (D) 72-hours with membrane-50, of blood fed and unfed resistant *Anopheles arabiensis* in the WHO tunnel test. Red dashed line depicts WHO mortality threshold of ≥ 80% mortality**
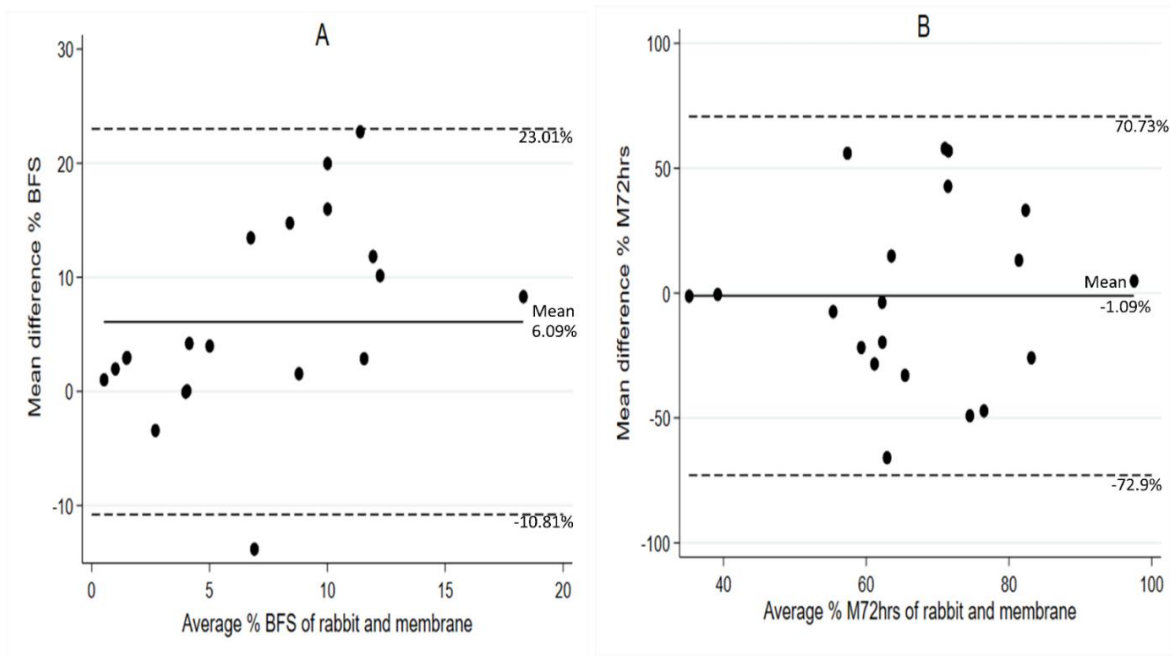
Appendix 5: Plot of mortality and blood feeding success for resistance *Anopheles arabiensis* by Bland and Altman

Plot (A) blood feeding success (BFS) and (B) 72-hours mortality (M72) for Interceptor® and Interceptor®G2 with rabbit or membrane bait with a density of 100-pyrethroid resistant *Anopheles arabiensis*and 12-hour exposure time in the WHO tunnel test. The average value for both methods is plotted on the x-axis and the mean difference between methods on the y-axis. The solid line in the middle shows mean difference with 95% confidence interval of the mean difference represented by the dashed lines.
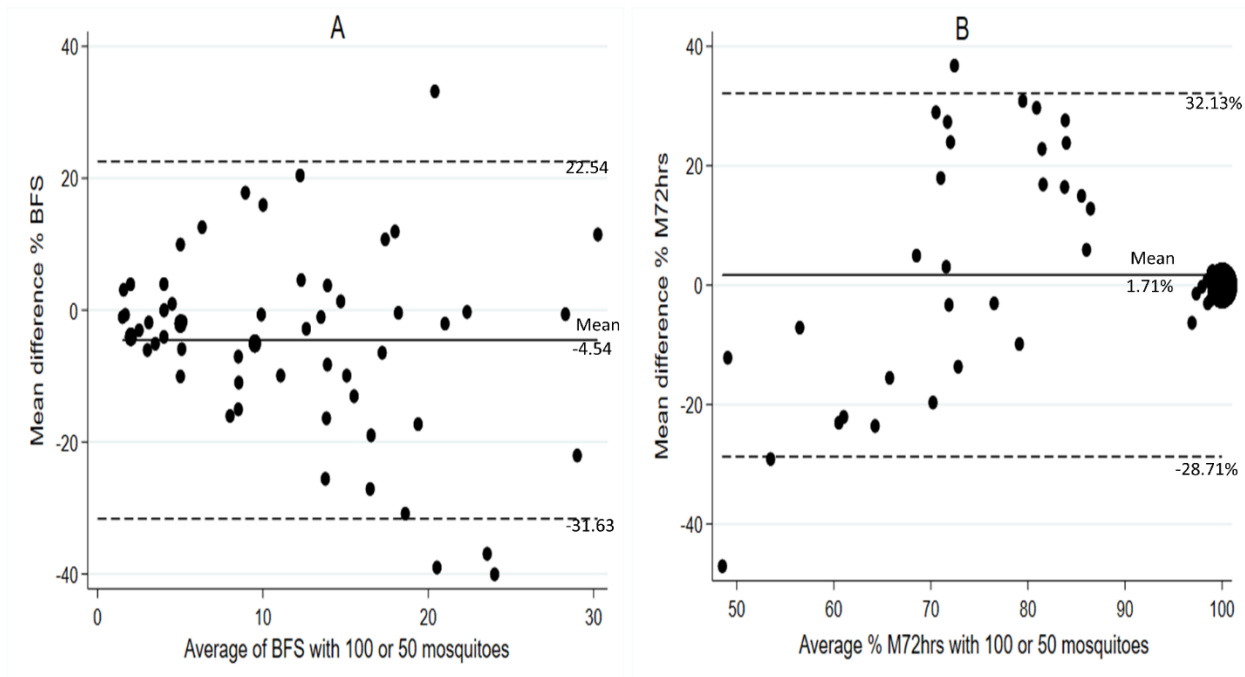
Appendix 6: Plot of mortality and blood feeding success for resistance and susceptible anopheles species by Bland and Altman

Plot of (A) blood feeding success (BFS) and (B) 72-hours mortality (M72) for Interceptor® with susceptible *Anopheles gambiae* and Interceptor®G2 with resistant *Anopheles arabiensis* using rabbit bait and a density of either 100 or 50 mosquitoes and a 12-hour exposure in the WHO tunnel test. The average value for both densities is plotted on the x-axis and the mean difference between densities on the y-axis. The solid line in the middle shows mean difference with 95% confidence interval of the mean difference represented by the dashed lines.
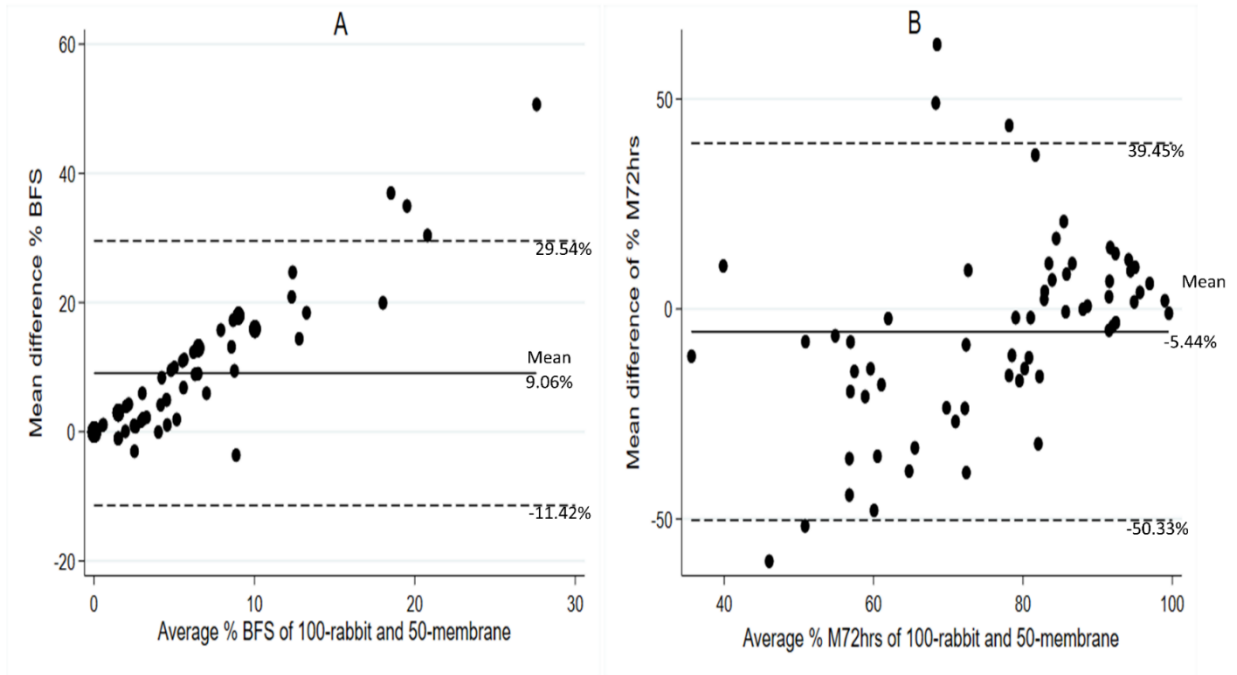
Appendix 7: Plot of mortality and blood feeding success for resistance *Anopheles arabiensis* with 50-membrane and 100-rabbit by Bland and Altman

Plot of (A) blood feeding success (BFS) and (B) 72-hours mortality (M72) for Interceptor® and Interceptor®G2 with resistant *Anopheles arabiensis* using rabbit bait and a density of 100 mosquitoes or membrane bait and a density of 50 mosquitoes with a 12-hour exposure time in the WHO tunnel test. The average value for both densities is plotted on the x-axis and the mean difference between densities on the y-axis. The solid line in the middle shows mean difference with 95% confidence interval of the mean difference represented by the dashed lines.

Appendix 8: Informed consent for human arm feeding in the WHO tunnel test

Informed consent for participants subjected to human arm feeding in WHO tunnel in English and Swahili

INFORMED CONSENT FORM FOR PARTICIPANTS

Name of Principle Investigator: Mr Dismas S. Kamande

Name of Institution: Nelson Mandela African Institution of science and Technology Collaboration Organization: Ifakara Health Institute

Name of Sponsor: Ifakara Health Institute, Bagamoyo, Tanzania

Project code: MSc 2

PART 1. INFORMATION SHEET FOR PARTICIPANTS

"Modified bioassay for testing insecticide treated nets: Comparison of three baits 1) Human arm 2) Membrane feeding (i.e. cattle blood) 3) Rabbit against strongly pyrethroid resistant *Anopheles arabieni*s in the WHO tunnel, Tanzania"

Introduction

My name is ……………….. <name of investigator >and I am student at Nelson Mandela African Institution of Science and Technology in collaboration with Ifakara Health Institute, Tanzania. I am conducting a study that aims at comparing the performance of alternative baits (human arm & membrane feeding) vs standard bait (Rabbit) for evaluation of ITNs bio-efficacy in WHO tunnel test. In additional, the study also aims at determining whether bait exposure time has effects on mortality and blood feed inhibition when evaluating ITNs bio-efficacy in WHO tunnel test. The type of ITNs used are brand from BASF Company, Germany. They are all approved for use by the World Health Organization pre-qualification for malaria control. The Government of Tanzania through the Tropical Pesticides Research Institute (TPRI) has also approved the nets for us to research in Tanzania.

Purpose of research

Malaria is one of the most important diseases in Tanzania. It is spread from one person to another through infected bites of certain mosquitoes. These mosquitoes normally bite at night. It has been shown that sleeping under mosquito nets can help to avoid getting bitten in the night. Furthermore, if the nets are treated with some chemicals that kill insects (insecticides), then they will prevent the bites and also kill the mosquitoes. Laboratory phase is the first proof of concept for bed nets towards killing infected mosquito. The current bioassay test has limitation including the use non-preferred bait, longer exposure time which can misreading the true effects of insecticide. Use of live animal is costing on caring the bait. This comparative study aims at investigating if the use of alternative bait (membrane and human arm) and the

exposure time have effects on mosquito mortality and blood feeding inhibition. Data generated will used to achieve the cost-effective means of evaluating ITNs bio-efficacy in WHO tunnel. Membrane as bait will be prepared using cattle blood which is less cost effective compare to live animal also free from welfare concern.

Information on study host/bait

The alternative bait (human arm) are considered to be gold standard bait when studing mosquito host seeking behaviour. Due to its ethical concern and risk of human on malaria once exposed to experiment it's less used. This comparative study need to include human in order to understand the different in performance among alternative bait and gold standard on evaluating ITNs bio-efficacy in WHO tunnel test. The tested nets during the entire experiments are from BASF Company and are safe for the participant. These products have been tested by the World Health Organisation and Tanzania Pesticide Research Institute and recommended as being safe and effective against malaria for all people to use.

Type of Research Intervention and procedures

- As a participant you will be asked to station your arm in the WHO tunnel chamber in short section for mosquito feeding between 19:30 hrs and 20:30 hrs.
- The work will be done in the laboratory with tunnel setup, reared mosquitoes do not have malaria and even if they bite you, you will not get a disease from them.
- The bed nets used are safer for use on humans and have been approved by the Tropical Pesticide Research Institute.
- You will be asked to wait for thirty minutes after exposure of 1hour arm feeding then allowed to go home.
- You will be asked to not smoke cigarettes or drink alcohol for the days or weeks that you are participating.
- You will need to take a malaria test every week that you are working on the study and sign a form to show that you have taken the test. The test will be paid by the study. If you are sick we will provide you with the correct medicine to treat malaria: ALU (artemesinin lufantrine) free of charge, and you will no longer allowed to take part in the study because you are sick.

Voluntary Participation

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

Risks

The risk of this study is you may be made uncomfortable by mosquito bites. You will be subjected to strong bite due to small chamber hence, we expect to receive many bites, although the bed nets have nine (9) holes in them. There is no chance of getting malaria while participating in the study. Although, if you suffer from fever, you should immediately seek for advice/assistance from the Ifakara Health Institute as per the contact details given below. Chlorfenapyr and alpha-cypermethrin, the two different insecticide classes subjected in the study has been tested before and has not been found to have any undue adverse effects in most people.. Some tingling or runny nose has been recorded in some people when nets are used for the first time when taken from its package. Based on the fact that only small part (i.e. human arm) of the whole body is

involved, less adverse event may happen i.e. itching is expected hence we will ask you if you have these symptoms report to one of our staff immediately at the contact details given below and we will provide you with all the necessary medical care.

Benefits

If you participate in this research, you will be entitle benefits: You will be given weekly screening for malaria and treatment if you get infected. Findings obtained will also be helping the recommendation of developing of cost effective bioassay test in WHO tunnel to support the timely evaluation of new products.

Compensation
You will receive Tshs.10,000 for your time away from home each 60minutes (1hour) of the experiment.
Who to contact

This proposal will be reviewed and approved by institution review board of IHI and National Institute of Medicacl Research, Tanzania, these boards are committee to make sure that research participants are protected from any harm.

In case you have any question or concern about this study please contact Mr Kamande Dismas S, research scientist/study investigator (Tel: +255 767 377878) and Dr Sarah Moore, senior research scientist/Study Core Supervisorr (Tel. No. +255 766468565) at IHI.

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

Should you wish to contact any of the above-named officials on phone, approach our field coordinators: Kamande Dismas (Telephone: +255 767377878) and Dickson Kobe (Telephone: +255746241575).

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

PART II: Certificate of Consent

I, …………………………………..clearly understand the aims of the project entitled **"Modified bioassay for testing insecticide treated nets: Comparison of three bait 1) Human arm 2) Membrane feeding (cattle blood) 3) Rabbit against strongly pyrethroid resistant *Anopheles arabieni*s in the WHO tunnel, Tanzania"**

and I agree to participate in the study.

During my participation in this studies, I understand that reared mosquitoes cannot infect me with malaria parasites.For adhering to ethical principal I therefore accept to undertake a weekly screening malaria diagnostic test (mRDT). I also understand that I am entitled to take free malaria prophylaxis and treatment for malaria in case I found to be infected with malaria parasites. I understand that I may revoke my consent and leave the study at any time.

Participant Name: _____

Participant Signature: _____Date _____(DD/MM/YY)

Witness Name: _____

Witness signature: _____Date _____ (DD/MM/YY)

If illiterate

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

Print name of witness_____AND Thumb print of participant

Signature of witness _____
Date _____(DD/MM/YY)

Statement by the study investigator / participant taking consent

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

1. The participant has been requested to place their arm under a WHO tunnel glass for 60minutes between 19:30 to 20:30 hours.
2. The participant has been requested to place his arm into a short section of tunnel and 100 mosquito will be released in long section of the WHO tunnel.
3. The participant has been informed that the mosquito used for arm feeding are laboratory reared, hence the bits cannot transmit malaria infection i.e. Mosquito age of 5-8 remarked as less likely to be infected and transmit disease.
4. The participant has been will be arriving in the testing building at 19:00pm, 1hour before commencement of the experiment.
5. Participant has been requested to refrain from smoking and consuming alcohol for the study duration.
6. Participant has been requested to take a malaria test every week that they are working on the study and sign a form to show that they have taken the test.
7. Participant has been informed that malaria testing and treatment will be paid for by the study.
8. Participant has been informed that if they test positive for malaria, they will not be allowed to take part in the study.
9. Participant will be reimbursed 10,000 Tsh per 60minutes (1hr) for work time taken up by the study

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

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

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____DD/MM/YYYY

Fomu ya ridhaa kwa washiriki wa kulisha mbu kwenye jaribio la handaki WHO -Swahili

FOMU YA RIDHAA KWA WASHIRIKI

Mtafiti Mkuu: Mr. Dismas S. Kamande

Jina la Taasisi: Nelson Mandela African Institution of Science and Technology, Tanzania

Mfadhili wa mradi: Ifakara Health Institute

Namba ya utambulisho wa mradi: MSc 2

SEHEMU 1: TAARIFA KWA MSHIRIKI

Tathmini ya bioassay ilioboreshwaa ya kupima net yenye dawa ya kuuwa mbu, mlinganisho wa chambo watatu kwenye handaki la WHO, Tanzania.

Utangulizi

Jina langu ni …………………………………………… (Jina la Mtafiti) na mwanafunzi wa shahada ya udhmiri katika chuo kikuu cha Nelson Mandela kwa kushirikiana na taasisi ya Afya ya Ifakara. Tunafanya tathmini ya njia mbadala ya kuweza kuhakiki ubora wa vyandarua vyenye dawa ya kuuwa mbu waambukizao malaria. Katika tathmini hii tutatumia chambo mbadala (i.e.utando wa bandia na mkono) tukiangalia matokeo ya vifo vya mbu na kushindwa kunywa damu kwa mbu pia kutathimini kama matokeo ya kufa na kushindwa kunyonya damu kwa mbu yanasababishwa na muda wa chambo kukaa ndani ya handaki la uchunguzi. Matokeo ya njia hii mpya yatalinganishwa na yale ya viwango vya WHO ya kutumia chambo sungura ili kuona kama itaweza kufanikisha matumizi ya gharama nafuu katika kupima ubora wa vyandarua kwa hatua ya awali (i.e.maabara) ambayo ni ushahidi wa kwanza katika kuhakiki ubora wa vyandarua kabla ya kupita kwenye hatua nyingine na mwisho kuwafikia watumiaji. Vyandarua vitakavyo husika ni vile vya interceptor®andinterceptor®G2vilivyo katika hali ya kuoshwa na kutokuoshwa na ambacho hakihitaji kurudiwa kuwekwa dawa. Serikali ya Tanzania kupitia Taasisi ya Utafiti na Udhibiti wa Viatilifu Tanzania (TPRI) wameidhinisha neti hizi ziweze kutumika kwa utafiti hapa Tanzania.

Lengo la Utafiti

Malaria bado ni tatizo kubwa hapa Tanzania. Malaria inaambukizwa kwa kuumwa na mbu jike wa aina ya Anopheles ambao huuma kuanzia wakati wa jioni baada ya jua kuzama na mapema asubuhi kunapo pambazuka. Tafiti zimethibitisha kuwa kwa kulala katika chandarua husaidia kuzuia kuumwa na mbu wakati wa usiku na mapema asubuhi. Tutatumia vyandarua vyenye viuatilifu kutoka makundi mawili tofauti ambayo inaongeza ufanisi hasa kukabiliana na mbu waliopatwa na usugu wa dawa ya kundi moja. Hivyo basi kuna utafiti huu wa kulinganisha chambo hawa mbadala na yule wa kiwango cha dunia katika handaki la WHO ni muhimu ili kurahisisha tathmini hiyo kuweza kufanyika katika nchi zote na kwa gharama nafuu ikitupa matokeo chanya. Tunapenda kukualika kama mshiriki kwenye utafiti huu, ambapo chambo mbadala wawili (i.e.utando wa bandia na mkono) watafanyiwa utafiti kwa kulinganishwa na chambo mwenye ubora (i.e.sungura) anaetumiwa kwa sasa na WHO kupitia nyenzo ya handaki (WHO

tunnel).Matokeo ya utafiti wetu ni idadi ya mbu kufa na idadi ya mbu kushindwa kunyonya damu. Aidha, kama mshiriki utatakiwa kuweka mkono wako katika shimo fupi la handaki katika chumba cha utafiti hapa VCPTU IHI_bagamoyomo. Neti zitakua zimekatwa vipande vya (i.e.25cm x 25cm). Kipande cha neti kitakua na matundu tisa ambayo mbu watatakiwa kupenya ili waweze kufikia chambo na kunywa damu. Baada ya muda wa mbu kukaa ndani ya handaki asubuhi yake mbu watakusanywa kwa chamba fupi na ndefu pamoja na kuangalia waliokunywa na wasiokunywa damu. wa mbUtatakiwa kukusanya mbu watakaoingia kwa kila asubuhi. Katika utafiti huu, vyandarua vitakavyotumika ni Interceptor® na interceptor®G2 vinavyotengenezwa na kiwanda cha BASF Germany.

Taarifa kuhusu chambo watakaotumika kwenye utafiti

Chambo mbadala watakao tumika katika utafiti huu 1) utandu wa bandia ambao utatokana na damu ya mnyama ng'ombe itakayo tunzwa katika friji iliopo chumba cha uchunguzi na itawekwa ndani ya feeder zitakazokua zimeunganishwa katika mfumo wa umeme ili kuhakikisha damu inakuwa na joto ili kuwavutia mbu kupata kunyonya damu. Kuongeza ufanisi wa matokeo, soksi ziliovaliwa na binadamu pamoja na gesi ya kabonidioxide ili kuwavutia mbu kunyonya damu zitatumika 2) Mkono wa ninadamu ambapo mshiriki atakae kubali kwa ridhaa yake atahusika kwenye zoezi la kunyonywa damu na wasio na maambukizi. Shughuli zote za utafti zitafanyika ndani ya chumba cha uchunguzi ambacho kitakua katika hali ya joto 27±5°C na unyevu wa karibu 80±10%. Taasisi ya taifa ya tiba (NIMR) na bodi ya usimamizi ubora wa utafiti ya ndani (IRB) wamejiridhisha na hatua zote zitakazofanyika ili kuhakiksha washiriki wote wa utafiti huu wanakua salama na madhara yoyote yanayoweza kujitokeza.

Aina ya njia za utafiti na taratibu zake

- Utatakiwa kuweka mkono ndani ya handaki kuanzia saa moja na nusu hadi saa mbili na nusu usiku baadaye mbu watabaki kwenye handaki mpaka saa moja na nusu asubuhi.
- Kazi itafanyika ndani ndani ya handaki. Mbu wasio na vimelea vya malaria wataachiwa ndani ya handaki la jaribio. Mbu hawa ni wakufungwa maabara hivyo hawana ugonjwa.
- Utatakiwa kuweka mkono ndani ya chemba fupi ya handaki la jaribio na mbu 100 wataachiwa ndani kupitia chemba ndefu.
- Vyandarua vitakavyotumika kwa tafiti hii ni salama kwa matumizi ya binadamu na vimepitishwa na Taasisi ya Utafiti na Udhibiti wa Viatilifu Tanzania (TPRI)
- Utatakiwa kupumzika kwa muda wa nusu saa (dakika 30) na baada ya hapo utaruhusiwa kuelekea mahali unapoishi.
- Hutatakiwa kuvuta sigara au kunywa pombe au kutumia kilevi cha aina yoyote muda utakayokuwa unashiriki katika tafiti hii
- Utahitajika kufanya vipimo vya malaria kila juma utakalokuwa unashiriki katika mradi huu na utasaini fomu kuonesha kuwa umeshachukua vipimo. Gharama za vipimo zitalipiwa na mradi. Kama utaumwa, utapatiwa matibabu sahihi ya malaria bure: ALU (artemesin lufantrine) bure bila malipo na hutaendelea kushiriki katika mradi kwakuwa unaumwa.

Ushiriki wa hiari

Ushiriki wako katika huu utafiti ni wa hiari. Ni chaguo lako kushiriki au kuacha. Unaweza kubadili uamuzi wakati wowote hata baadae na kusimama kushiriki hata kama ulikubali hapo mwanzo. Hii haitaathiri ufanyaji kazi wako na IHI. Ni chaguo lako na haki zako zote zitaheshimiwa.

Hatari

Hatarishi ni unaweza usijisikie vizuri kung'atwa na mbu na kupata maumivu ya mkono ambayo yanaweza kupelekea kupatwa na hali ya tofauti mwilini. Hivyo tunakuhakikishia kukupa ushirikiano kwa hali yoyote ya afya itakayo jitokeza katika kipindi chote utakapo kuwa katika majaribio. Tunapenda kukuhakikishia kuwa hakuna uwezekano wa kuambukizwa malaria wakati wa ushiriki katika utafiti huu. Ikitokea ukapata maambukizi, itakupasa kuwasiliana na msimamizi mkuu wa utafiti huu kati ya chuo kikuu cha Nelson Mandela na mshiriki wake taasisi ya Ifakara ambao mawasiliano yao yameambatishwa katika fomu hii msaada zaidi.

Faida kwa mshiriki

Kwa kushiriki utafiti huu, utapata faida zifuatazo; Kila wiki utapimwa kwa ajili ya kuangali kama umepata maambukizi ya malaria. Ushiriki wako utatusaidia kufanikisha upatikanaji wa taarifa zitakazo tusaidia kupata chambo wa uhakika na muda umuhimu wa ma muda ili kufanikisha gharama nafuu katika kufanya tathmini ya ubora wa vyandarua kwa hatua ya kwanza kupitia handaki ya WHO. Njia hii itasaidia kuharakisha zoezi la uhakiki wa ubora wa vyandarua ambavyo vitakiwa kutumika kwa matumizi ya kuzuia maambukizi ya malaria kwa binadamu wengine.

Makubaliano
Utalipwa Tshs 10,000 kwa kila usiku utakapokuwa nje ya nyumba yako kwa ajili ya utafiti.

Nani wa kuwasiliana nae

Pendekezo la mradi huu limepitiwa na kupata kibali kutoka Bodi ya maadili ya Taasisi ya Afya Ifakara (IHI) na bodi ya maadili ya Taasisi ya Taifa ya utafiti wa magonjwa ya binadamu (NIMR) ambayo ni kamati yenye jukumu la kuhakikisha kila mshiriki wa utafiti analindwa na hatari zozote.

Kama una swali lolote kuhusiana na utafiti huu, unaweza kuwasiliana na Mr Kamande Dismas (Tel: +255767377878) na Dr Sarah Moore wa taasisi ya afya Ifakara (IHI) kupitia namba (Tel: +255766468565).

Hata hivyo kama hujaridhika na majibu unayopewa na timu ya utafiti, unaweza kuwasiliana na mwakilishi wa bodi ya maadili ya Taasisi ya Afya Ifakara Dr Mwifadhi Mrisho (+255 788 766 676) au Ms Sia Malekia, (+255 754 499 293) NIMR.

Kwa tatizo lolote wasiliana na wahusika ambao ni wafanya kazi wetu watakao simamia mradi huu Ahmad Bakari Hassan kwa namba zifuatazo+255683075207.

Kwa kumbukumbu zako za ushiriki katika kazi hii utabaki na kopi ya fomu moja uliyojaza na kusainiwa na m.

SEHEMU II: Cheti cha Ushiriki

Mimi,…………………………………………Nimeelewa vizuri madhmuni ya utafiti huu unaoitwa **"Tathmini ya bioassay ilioboreshwaa ya kupima net yenye dawa ya kuuwa mbu, mlinganisho wa chambo watatu kwenye handaki la WHO, Tanzania"** na ninakubali kushiriki katika utafiti. Wakati wa ushiriki wangu katika utafiti huu, Ninafahamu kuwa ninaweza kuumwa na mbu na kupata maambukizi ya malaria.Pia naelewa kuwa nitafanya kazi usiku kwa muda wa saa moja kwa kuweka mkono ndani ya handaki, hivyo kuwa katika uhatarishi wa kuumwa na mbu wanaoweza nisababishia malaria. Hivyo nakubali kuwa tayari kwa ajili ya upimaji wa malaria kila wiki kwa kutumia kipimo kiitwacho mRDT.

Naelewa pia yanipasa kumeza dawa za kuzuia uambukizi wa malaria (prophylaxis) na pia nipo katika utaratibu wa kupata matibabu ya malaria bure inapotokea nimepata maambukizi. Ninafahamu pia kuwa ninaweza kubadilisha maamuzi kuhusu ushiriki wangu kwa kujitoa wakati wowote.

Jina la Mshiriki:_____

Sahihi ya Mshiriki: _____Tarehe_____SIKU/MWEZI/MWAKA

Jina la shahidi: _____
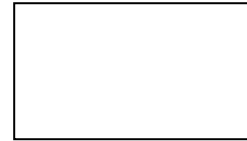
Sahihi ya shahidi:_____Tarehe_____ SIKU/MWEZI/MWAKA

Kama hajui kusoma wala kuandika;

Nimeshuhudia usomaji sahihiwa fomu ya ridhaa kwa mshiriki muhimu, na mshiriki amepata nafasi ya kuuliza maswali. Ninathibitisha mshiriki ametoa ridhaa kwa uhuru.

Andika jina la shahidi_____NA dole gumba la mshiriki

Sahihi ya shahidi _____

Tarehe_____(DD/MM/YY)

Maneno ya mtafiti/ mtu anayechukua ridhaa

Nimesoma kwa usahihi karatasi ya taarifa kwa mshiriki na kwa uwezo wangu wote nimehakikisha kuwa mshiriki ameelewa kuwa yafuatayo yatafanyika:

1. Mshiriki ametaarifiwa kuwa ataweka mkono ndani ya handaki la WHO yenye kipande cha chandarua chenye matundu tisa kuanzia kuanzia saa moja na nusu jioni hadi saa mbili na nusu usiku.
2. Mshiriki ametaarifiwa kuwa kazi itafanyika kwenye handaki la WHO ambapo mkono tu nido sehemu ya mwili itakayong'atwa na mbu ivyo uwezekano mdogo kuambukizwa ugonjwa wa malaria.
3. Mshiriki ameombwa kuingiza mkono kupitia chemba fupi ya handaki na kisha mbu wataachiwa kuipitia chemba ndefu ya handaki hilo.
4. Mshiriki ametaarifiwa kuwa vipande vya vyandarua vyenye dawa ni salama na vimethibitishwa na Taasisi ya Utafiti na Udhibiti wa Viatilifu Tanzania (TPRI)
5. Mshiriki ametaarifiwa kuwa atatakiwa kupumzika kwa muda wa nusu saa mara baada ya kumaliza kushiriki katika jaribio la kuweka mkono ndani ya chemba ya handaki.
6. Mshiriki ametaarifiwa kuwa atatakiwa kutokuvuta sigara au kunywa kilevi cha aina yoyote kwa siku au majuma atakayokuwa anashiriki kwenye utafiti huu.
7. Mshiriki ametaarifiwa kuwa atatakiwa kufanya uchunguzi wa vipimo vya malaria mara moja kila juma na kusaini fomu kuonesha kuwa amefanya vipimo hivyo.
8. Mshiriki ameambiwa kuwa vipimo vya uchunguzi na matibabu ya ugonjwa wa malaria vitalipiwa na mradi.
9. Mshiriki ameambiwa kuwa endapo atapatwa na maambukizi ya malaria, hataendelea kushiriki katika mradi.
10. Mshiriki atalipwa kiasi cha Tshs 10,000 kwa kila usiku anaoshiriki katika kazi.

Ninathibitisha kuwa mshiriki amepewa nafasi ya kuuliza maswali kuhusu mradi na maswali yote yaliyoulizwa na mshiriki yamejibiwa sahihi na kwa uwezo wangu. Ninathibitisha kuwa mshiriki hajalazimishwa kutoa ridhaa ya kushiriki utafiti huu na kwamba ridhaa imetolewa kwa hiari na kwa uhuru.

Ninathibitisha kuwa nakala ya fomu hii amepatiwa mshiriki

Andika Jina la mtafiti/ Mtu anayechukua ridhaa_____

Sahihi ya mtafiti/mtu anayechukua ridhaa_____

Tarehe _____DD/MM/YYYY

Appendix 9: Permission to publish was granted from NIMR

UNITED REPUBLIC OF TANZANIA

MINISTRY OF HEALTH

NATIONAL INSTITUTE FOR MEDICAL RESEARCH

In reply please quote:

Ref. No: NIMR/HQ/P.12 VOL XXXIV/39   Date: 11th April 2022

Dismas Kamande
Ifakara Health Institute
Bagamoyo Branch
P.O. Box 74
BAGAMOYO.

Dear Dismas Kamande,

RE: PERMISSION TO PUBLISH

Reference is made to your request to publish data from a study with ethical clearance number NIMR/GQ/R.8a/Vol.IX/3893.

1. Permission has been granted to publish a manuscript titled: "**Alternative versions of the World Health Organization (WHO) tunnel test considering the effect of alternative hosts, exposure time, and mosquito density for higher throughput 66 evaluation of insecticide-treated nets (ITNs)**" by authors: Dismas S. Kamande, Olukayode G. Odufuwa, Emmanuel Mbuba, Ahmad B. Mpelepele, Stephen G. Mbwambo, Ummi A. Kibondo, Jason Moore, Mgeni M. Tambwe, Lorenz M. Hofer and Sarah J. Moore.

2. Please submit an electronic copy of the published manuscript to the National Institute for Medical Research through email publications@nimr.or.tz.

Dr. Ndekya Maria Oriyo

DIRECTOR OF RESEARCH INFORMATION, TECHNOLOGY AND COMMUNICATION

RESEARCH OUTPUT

insects   MDPI

Modified World Health Organization (WHO) tunnel test for higher throughput evaluation of insecticide-treated nets (ITNs) considering the effect of alternative hosts, exposure time, and mosquito density

Dismas S. Kamande [1,2*], Olukayode G. Odufuwa [2,3,4,5], Emmanuel Mbuba [2,4,5], Lorenz Hofer [4,5], Sarah J. Moore [1,2,4,5]

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[2]Vector Control Product Testing Unit (VCPTU) Ifakara Health Institute, Environmental Health, and Ecological Sciences, P.O. Box 74, Bagamoyo, Tanzania; embuba@ihi.or.tz; smoore@ihi.or.tz

[3]MRC International Statistics and Epidemiology Group, London School of Hygiene and Tropical Medicine, London; oodufuwa@ihi.or.tz

[4]Vector Biology Unit, Department of Epidemiology and Public Health, Swiss Tropical & Public Health, Institute, Kreuzstrasse 2, 4123 Allschwill, Basel, Switzerland; lorenz.hofer@swisstph.ch

[5]University of Basel, Petersplatz 1, 4001, Basel, Switzerland

Correspondence: dkamande@ihi.or.tz

Simple Summary: Membrane feeding assays have been widely used in malaria transmission research and insectary colony maintenance. Here, we investigate whether a membrane feeder can replace animal baits for evaluating Insecticide Treated Net (ITNs) bio-efficacy in the World Health Organization (WHO) tunnel test. The effect of 1) alternative baits, 2) exposure time, and 3) mosquito density on the endpoints of mosquito mortality and feeding inhibition or feeding success was investigated. Our results show that similar mortality at 24- or 72-hours is estimated using either a membrane feeder or a rabbit bait with an overnight (12 hours) exposure. However, the membrane measured higher blood feeding inhibition than the rabbit likely due absence of host cues, notably carbon dioxide. Therefore, the membrane feeder may be used instead of an animal bait to accurately test mortality endpoints in WHO tunnel tests. Experimental results demonstrated that using 50 or 100 mosquitoes per replicate measure the same for mortality and feeding inhibition endpoints with an animal bait. Therefore, WHO tunnel tests may be run with lower mosquito densities. This will reduce strain on insectaries to produce sufficient mosquitoes to meet the large sample sizes needed for bio-efficacy durability monitoring of chlorfenapyr ITNs that mustbe evaluated in "free-flying" bioassays.

Keywords: WHO tunnel test, Insecticide Treated Nets, ITNs, Interceptor, InterceptorG2, membrane, human arm, rabbit, bioassay, bio-efficacy, mosquito, *Anopheles.*

Output 2: Poster presentation

Modified World Health Organization (WHO) Tunnel Test for Higher Throughput Evaluation of Insecticide-Treated Nets (ITNs)

ISO 9001:2015 certified

IFAKARA HEALTH INSTITUTE
research | training | services

Dismas S. Kamande, Olukayode G. Odufuwa, Emmanuel Mbuba and Sarah J. Moore

Introduction

- Durability bio-efficacy monitoring is critical process for measuring efficacy of population ITNs with single or dual ITNs.

- WHO tunnel test a "free flying" is reliable laboratory bioassay used for testing of insecticide-treated nets (ITNs) bio-efficacy

- Here, a comparison study was conducted with alternative bioassay against the current gold standard WHO tunnel test as outlined in the guideline for ITN evaluation
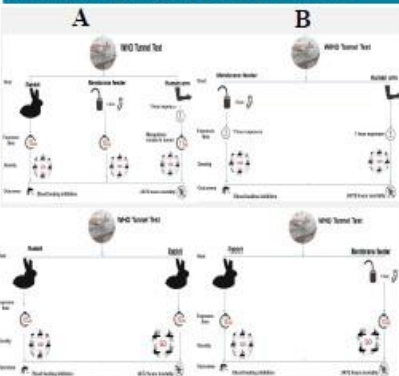
Methods: Experimental set-up and conduct

- **Figure1**: Standard WHO tunnel bioassay conducted (A) with different host (B) Reduced exposure time (C) Reduced mosquito density (D) Replacement of rabbit bait

Methods: Conduct of WHO tunnel test

- Experiments were conducted with a minimum of 5 replicates per net type, per permutation.

- All procedures for conducts were performed as per the standard WHO tunnel test procedure

- Outcomes were blood feeding success (BFS) or blood feeding inhibition (BFI), mortality at 24-h (M24), and mortality at 72-h (M72).

Results: Effects of alternative host and mosquito density

Table 1: Using 50 or 100 mosquito with rabbit as bait measures similar outcomes in WHO tunnel test

Density	BFS			M72		
	% (95% CI)	OR (95%CI)	P-value	% (95% CI)	OR (95%CI)	P-value
Susceptible An. gambiae with Interceptor®						
100 Mosquitoes	5.80 (3.4-8.2)	1		99.1 (98.6 - 99.6)	1	
50 Mosquitoes	9.10 (6.6-11.6)	2.35 (0.80-6.92)	0.12	99.6 (99.3 - 99.9)	1.80 (0.43-7.54)	0.42
Resistant An. arabiensis with Interceptor®G2						
100 Mosquitoes	12.50 (8.9-16.0)	1		73.9 (66.7 - 81.2)	1	
50 Mosquitoes	18.30 (13.3-23.2)	1.54 (0.74-3.22)	0.25	70.0 (67.0 - 73.1)	0.65 (0.25-1.67)	0.38

Table 2: Membrane assay with 50 mosquito measures 72hours mortality similar to gold standard WHO tunnel test

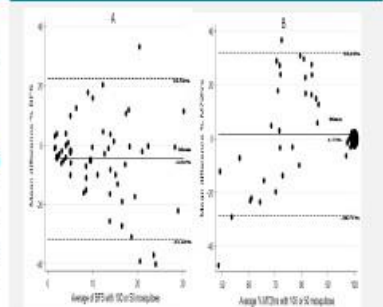| Bioassay	BFS OR (95%CI)	P-value	M24 OR (95%CI)	P-value	M72 OR (95%CI)	P-value
Interceptor®						
100 Rabbit	1		1		1	
50 Membrane	0.19 (0.08-0.45)	< 0.001	0.39 (0.10-1.61)	0.19	0.54 (0.14-2.06)	0.37
Interceptor® G2						
100 Rabbit	1		1		1	
50 Membrane	0.17 (0.09-0.30)	< 0.001	1.10 (0.51-2.36)	0.81	1.50 (0.76-2.98)	0.25

Results: Agreement between bioassays

Figure 3: Bland & Altman measure of agreement for reduced mosquito density (A) Blood feeding success (BFS) (B) Mortality at 72hours

Conclusion

- Using 50 or 100 mosquitoes gives similar results with no systematic bias
  - Reducing the number of mosquitoes
  - Larger number of net samples can be tested
- Membrane or rabbit estimate similar mortality
  - Improve blood feeding success is critical
  - BFS can affect mortality estimates
- Conducted of similar experiment in other testing facility is needed

Acknowledgement

- Ifakara Health Institute
- Nelson Mandela African Institution of Science and Technology

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