Long-Lasting Insecticidal Nets retain bio-efficacy after five years of storage: implications for malaria control programmes

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Abstract

Background: Long Lasting Insecticidal Nets (LLINs) are the most sustainable and effective malaria control tool currently available. Global targets are for 80% of the population living in malaria endemic areas to have access to (own) and use a LLIN. However, current access to LLINs in endemic areas is 56% due to system inefficiencies and budget limitations. Thus, cost-effective approaches to maximize access of effective LLINs in endemic areas are required. This study evaluated whether LLINs that had been stored for five years under manufacturer's recommended conditions may be optimally effective against Anopheles mosquitoes, to inform malaria control programs and governments on the periods over which LLINs may be stored between distributions, in an effort to maximise use of available LLINs.

Methods: Standard World Health Organization (WHO) bioassays (cone and tunnel test) were used to evaluate the bio-efficacy and wash resistance of Olyset® and DawaPlus® 2.0 (rebranded Tsara® Soft) LLINs after five years of storage at 25°C - 33.4°C and 40% - 100% relative humidity. In addition a small scale, Ifakara Ambient Chamber tests (I-ACT) were conducted to compare the bio-efficacy of one long stored LLINs to one new LLIN of the same brand, washed or unwashed. LLINs were evaluated using laboratory reared fully susceptible Anopheles gambiae s.s. (Ifakara) and pyrethroid resistant Anopheles arabiensis (Kingani).

Results: After five years of storage, both unwashed and washed, Olyset® and DawaPlus® 2.0 LLINs passed WHO bio-efficacy criteria on knockdown (KD60) ≥95%, 24-hour mortality ≥80% and ≥90% blood-feeding inhibition in WHO assays against susceptible An. gambiae s.s. DawaPlus® 2.0 LLINs also passed combined WHO bioassay criteria against resistant An. arabiensis. Confirmatory I-ACT tests using whole nets demonstrated that long stored LLINs showed higher efficacy than new LLINs on both feeding inhibition and mortality endpoints against resistant strains.

Conclusions: Even after long-term storage of around 5 years, Olyset® and DawaPlus® 2.0 LLINs remain efficacious against susceptible Anopheles mosquitoes at optimal storage range of 25°C - 33.4°C for temperature and 40% - 100% relative humidity measured by standard WHO methods.

Background

Long lasting Insecticidal Nets (LLINs) remain the most sustainable and effective malaria control tool available in endemic countries [1], despite insecticide resistance [2]. Approximately 663 million cases of malaria were prevented by LLINs since the year 2000, representing 68% of the total cases averted by all interventions used for malaria control [3]. In 2007, the mass distribution of LLINs was recommended by the World Health Organisation (WHO) as the core element of the global malaria strategy for malaria vector control in endemic areas [4, 5]. Between 2008 to 2016, more than one billion LLINs were distributed in Africa through mass campaigns [6]. The wide scale up of LLIN distribution has led to a significant reduction of malaria morbidity and mortality [7]. Although, before a brand of LLIN can be listed as a potential product for mass campaign by the WHO, it must have undergone rigorous testing from

laboratory to field testing [8]. Currently, there are twenty brands of LLINs that are prequalified by the WHO for use in national distribution campaigns [9]. These LLINs are expected to retain their insecticidal activity for at least 3 years (20 washes), by killing mosquitoes and preventing mosquito bites, and confer both personal and community protection from vector borne diseases [8].

The public health benefit of LLINs is attained through sustained high net access at the community level, which is referred to as universal coverage [10]. Currently, the global target for population access to LLINs is > 80%, and is referred to as the minimum operational effectiveness coverage level that will translate to the community effect of LLINs [11]. Operationally, this is defined as one net used per two people (defacto) in the population [12, 13].

The governments of malaria endemic countries and international donors such as Global Fund, U.S. President's Malaria Initiative (PMI) as well as non-governmental organisations (NGOs) have been providing funds for procurement of LLINs and related logistics to ensure high access to LLINs through multiple channels [14, 15]. Nevertheless, current access to LLINs is 56% in endemic areas [16]. Even shortly after mass distribution campaigns of LLINs, population access rarely exceeds 80% [6, 12, 17]. In Tanzania, access to LLINs is 50%, [16] and trends in malaria burden can be seen in Tanzania where malaria declines in years after mass campaigns and then increases in the following years as LLINs wear out. Insufficient access to LLINs is mainly due to long intervals between net distribution campaigns, population growth, inadequate funds and budget limitations on malaria control programs [5, 16–18]. Increasing access to LLINs through cost-effective solutions remains a critical concern and a number of strategies are being explored for "keep-up campaigns" to retain high LLIN access [13].

It is known that the correct storage of nets is important to retain their bio-efficacy, before and during mass distribution campaigns. Exposure of LLINs to direct sunlight [19] and storage at high temperature will degrade the pyrethroid insecticides used on LLINs [19, 20] and guidance on the correct storage conditions for LLINs before and during distributions is available [21]. However, there is limited information on the maximum storage period for LLINs before they are no longer suitable for use. Therefore, this study evaluated the bio-efficacy and wash resistance of DawaPlus® 2.0 (currently rebranded Tsara® soft) and Olyset® LLINs that have been stored for more than five years (long storage LLINs) under optimal conditions of 25°C – 33°C and 40% – 100% relative humidity.

Methods

Study design

Two brands of LLINs: Olyset® and DawaPlus® 2.0 that were stored for more than five years under recommended conditions were evaluated. The study was conducted in two stages. First, through a randomized double blinded, bio-efficacy evaluation of LLINs using standard WHO assays [8]. This was followed by partially randomized double blinded semi-

field tests to compare the bio-efficacy of long stored LLINs against the new LLINs of the same brand using the Ifakara Ambient Chamber tests (I-ACT) [22]. Untreated Safi Net was used as a negative control in all tests to monitor the quality of the experiment.

Test facility

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

Test nets

Olyset® is a high-density mono-filament polyethylene (HPDE) LLIN, incorporated with 20 g/kg (±3 g/kg), 2% w/w of permethrin (corresponding to 1000 mg/m^2). Olyset® is manufactured by A to Z Textile Mills Ltd, Arusha, Tanzania. DawaPlus® 2.0 LLIN (currently Tsara® soft) is a deltamethrin-coated LLIN. The target dose of deltamethrin incorporated on a knitted multi-filament polyester fiber is 2.0 g/kg ± 25% with 100-denier yarn (corresponding to 80 mg/m^2) deltamethrin. Tsara soft is manufactured by NRS Moon Netting FZE [9]. Untreated Safi Net is made of polyester fibres, manufactured by A to Z Textile Mills Ltd, Arusha, Tanzania. All nets were double sized and coded by an independent technician, to allow blinding of investigators and participants. All test nets have WHO-PQ listing [9].

Net Storage conditions

Olyset® and DawaPlus® 2.0 LLINs were stored for approximately five years in the Ifakara Health Institute (IHI-Bagamoyo) storage facility and are denoted in this study as long storage (LS) nets. All nets were received directly under similar conditions from the manufacturer and were manufactured shortly before shipping for the purpose of product evaluation. LS Olyset® LLINs with batch number L2605 were manufactured in May, 2013 and logged into the IHI-Bagamoyo storage facility on 4th June, 2013. LS DawaPlus® 2.0

LLINs were regular production manufactured in November, 2013 and were logged into the IHI-Bagamoyo storage facility on 4th December, 2013.

The new Olyset® LLINs were manufactured in 2017 with batch number 7X15BZS, and were logged into the IHI-Bagamoyo storage facility on 22nd December, 2018. The new DawaPlus® 2.0 LLINs were test series manufactured on March, 2018, with batch number 18SPL005, were shipped from the manufacturer on 15th May, 2018 and logged into the IHI-Bagamoyo storage facility on 1st June, 2018. All nets were stored and maintained at an average temperature of 29°C [25°C - 33.4°C] and 40% - 100% relative humidity in the IHI-Bagamoyo storage facility. Temperature was recorded and logged each afternoon at 14:00 hours which coincides with peak temperatures.

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

LLINs preparation and washing procedures for WHO assays

Eight LLINs (4 nets of each brand) were selected at random from the same product batch. LLINs were coded, cut into pieces (25cm x 25cm) and washed at 1, 3, 5, 10, 15 and 20 times following WHO standard procedures for laboratory testing (phase I) [8] and a 1 day washing interval based on the reported regeneration time for both products [23].

LLINs preparation and washing procedures for I-ACT assays

Eight LLINs (2 old and 2 new DawaPlus® 2.0 and 2 old and 2 new Olyset®) and 2-untreated nets were randomly selected from their product batches and coded. Two LLINs of each brand were washed 20 times as per WHO small-scale field trials (phase II) washing

procedures, used as a standard procedure to simulate aging of nets under user conditions [8], while the other two were unwashed. All washed, unwashed and un-treated nets were deliberately holed 6 times with 4cm by 4cm with one hole on each width and two holes on each length side, 75 cm from the top of the net as per WHO procedures [8]

Test systems

The study used *Anopheles (An.) gambiae* s.s. (Ifakara strain) fully susceptible to all classes of insecticides and *An. arabiensis* (Kingani strain) strongly resistant to all pyrethroids including, deltamethrin and permethrin (<20% mortality with WHO discriminating doses, through metabolic CYP450 mechanism). In WHO cone bioassays, nulliparous 3-5 day old female sugar-fed mosquitoes were used while in the tunnel test and I-ACT, nulliparous 5-8 day old female mosquitoes sugar-starved for eight hours were used. The VCPTU mosquito colonies are maintained at 27°C ± 5°C and 40% - 100% relative humidity with access to 10% sucrose *ad libitum* supplemented by membrane feeding using cow blood for the purposes of egg laying following MR4 guidelines [24].

WHO assays

Cone bioassays were conducted on unwashed and washed long storage LLINs, with four LLINs of each brand tested per condition. Five mosquitoes were exposed for three minutes per cone on each net replicate. Long storage LLINs that failed to meet WHO cone bioassay threshold criteria (Table 1), were subjected to WHO tunnel test. Each test replicate and associated control was tested with both the susceptible and resistant strain as per WHO guideline [8].

I-ACT assays

The I-ACT was used as an intermediate between laboratory and experimental hut tests [22]. One LLIN per condition (unwashed or 20 times washed) was tested as a confirmation of the WHO laboratory bioassay findings. Each LLIN or control was randomly assigned to one of the ten testing chambers of the I-ACT (Figure 1a). At 21:00 hours, sleepers released 30 *An. gambiae and* 30 *An. arabiensis,* in each testing chamber. Mosquitoes were lightly dusted

with fluorescent powder (SWADA, Cheshire, United Kingdom) to distinguish the strain as they are morphologically identical. At 06:00 AM, mosquitoes were collected into paper cups using a mouth aspirator. Mosquitoes were scored immediately after collection by strain and four categories: 1) dead and unfed, 2) dead and blood-fed, 3) alive and unfed or 4) alive and blood-fed. Mosquitoes were then held in the testing laboratory at 27°C ± 5°C and 40% - 100% relative humidity with access to 10% sugar solution. After 24-hours, the proportion of mosquitoes in each of the four categories was again scored using the above criteria. Following each night of the experiment, test nets were re-packed in their respective bags, chambers were cleaned and bed sheets were washed. LLINs remained fixed to their respective chambers while volunteers rotated nightly for ten experimental nights so that each volunteer tested each net type once. This was done to account for difference between human attractiveness to mosquitoes that might affect the proportion of mosquitoes blood feeding. Acceptable mortality was ≤ 10% or ≥50% blood-feeding success in control [8] Table 1, Figure 1).

Figure 1. I-ACT Assay

A- Schematic diagram of the Ifakara Ambient Chamber Test (I-ACT with10 chambers); B- I-ACT at IHI-Bagamoyo branch; C- Sleeper releasing mosquitoes within chamber outside the net; D– volunteer (sleeper) sleeping in side net within a chamber; E- Sleeper collecting mosquitoes using mouth aspirator (siphon) inside net within chamber,

F- Sleeper collecting mosquitoes using siphon outside net within chamber.

Table 1. The summary of experimental design on WHO and I-ACT Bioassays

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

Data management and analysis

Data were recorded onto paper forms, double entered into Microsoft excel 2013 and cleaned prior to analysis. Data analysis was performed using STATA 13.1.

Descriptive statistics were used for WHO cone and tunnel tests. For the I-ACT, both descriptive statistics and binomial logistic regression with mixed effects were conducted. The outcome measures were 24-hour mortality and blood-feeding inhibition. Model fit was tested using AIC [25]. For the model with mortality as the outcome, the best fitting model had treatment and volunteer as fixed effect and day as a random effect while best model

with feeding success as the outcome had treatment as a fixed effect, with both volunteer and day as random effects.

Results

WHO assays with susceptible An. gambiae s.s (Ifakara strain)

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

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

WHO assays with resistant An. arabiensis (Kingani strain)

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

LS DawaPlus® 2.0 LLINs fulfilled WHO bioefficacy criteria up to 20 washes based on the combined WHO Cone bioassay and tunnel test against resistant An. arabiensis (Table 2). LS DawaPlus® 2.0 LLIN, either demonstrated > 95% KD60 (Fig. 3a) in cone bioassay or > 90% feeding inhibition (Fig. 3d). It did not demonstrate 80% 24-hour mortality up to 20 washes (Fig. 3c) in both cone and tunnel tests.

Table 2

WHO bio-assays results against susceptible An. gambiae s.s and resistant An. arabiensis

Test system	Test item	Washes	Cone test (N = 80)		WHO Tunnel test (N = 100)		Pass/Fail WHO efficacy criteria (2013)
			% KD60 [95% CI]	% 24-HRS Mortality [95% CI]	% Feeding Inhibition [95% CI]	% 24-HRS Mortality [95% CI]	
Susceptible Anopheles gambiae (Ifakara strain)	Olyset®	0	100	03.75 [01.47–06.03]	-	-	Pass
		1	100	31.64 [29.40-33.89]	-	-	Pass
		3	96.25 [95.06–97.44]	01.25 [00.06–02.44]	-	-	Pass
		5	93.75 [89.24–98.26]	0	-	-	Pass
		10	93.75 [92.56–94.94]	02.50 [01.12–03.88]	-	-	Pass
		15	83.75 [79.68–87.82]	01.25 [00.06–02.44]	100	90.91	Pass
		20	75.00 [69.85–80.15]	03.75 [01.47–06.03]	96.00	51.52	Pass
	DawaPlus®	0	100	92.50 [90.12–94.88]	-	-	Pass
		1	100	100	-	-	Pass
		3	100	98.75 [97.56–99.94]	-	-	Pass
		5	100	97.50 [96.12–98.88]	-	-	Pass
		10	100	96.25 [92.68–99.80]	-	-	Pass
		15	100	93.75 [87.79–99.71]	-	-	Pass
		20	100	100	-	-	Pass
Resistant Anopheles arabiensis (Kingani)	Olyset®	0	38.75 [34.68–42.82]	0	-	-	-
		1	25.00 [19.85–30.15]	01.25 [00.06–2.44]	-	-	-
		3	23.75 [19.68–27.82]	03.75 [01.47–6.03]	96.00	09.18	Pass
		5		03.75 [02.56–4.94]	87.00	12.24	Fail
		10			87.00	14.29	Fail
		15			92.00	19.39	Pass
		20			88.00	22.45	Fail

		51.25 [47.18–55.32]	01.25 [00.06–2.44]			
		36.25 [33.25–39.25]	0			
		53.75 [40.79–66.71]	0			
		58.75 [57.56–59.94]				
DawaPlus®	0	50.00 [44.85–55.15]	10.00 [05.64–14.35]	-	-	-
	1	63.75 [61.47–66.03]	11.25 [06.74–15.76]	-	-	-
	3	67.50 [63.37–71.63]	16.25 [15.06–17.44]	90.00	61.62	Pass
	5	91.25 [87.68–94.82]	36.25 [31.34–41.16]	94.00	56.57	Pass
	10	100	23.75 [19.68–27.82]	97.00	78.79	Pass
	15	97.50 [96.12–98.88]	05.00 [01.63–08.37]	68.00	51.52	Pass
	20	93.75 [89.24–98.26]	12.50 [09.42–15.58]	93.00	41.41	Pass

N = number of mosquitoes released on each test

I-ACT results against susceptible An. gambiae s.s (Ifakara strain)

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

I-ACT results against Resistant An. arabiensis (Kingani)

Against resistant An. arabiensis, unwashed LS Olyset® and unwashed LS DawaPlus® 2.0 exceeded the WHO bio-efficacy criteria for tunnel tests on feeding inhibition (≥ 90%). All the net types and condition failed to meet WHO bioefficacy criteria on 24-hour mortality (≥ 80%) against the resistant strain. Unwashed and 20x washed Olyset® and DawaPlus® 2.0 LS nets performed in a similar way to new nets of the same brand and washing status on both endpoints showing almost identical mortality and feeding inhibition (Table 3).

As was observed with the susceptible strain, on the mortality endpoint, LS unwashed Olyset® marginally outperformed the new unwashed Olyset® 63.40% [47.83−78.97] vs 50.31% [33.42−67.19], Odds ratio 0.49 [95% CI: 0.33−0.72], p < 0.0001. On the feeding inhibition endpoint, LS unwashed DawaPlus® 2.0 marginally outperformed the new unwashed Tsara® Soft: 91.57% [88.72−94.41] vs 81.78% [75.49−88.07], OR 2.55 [1.61−4.06], p < 0.0001. Additionally, on the feeding inhibition endpoint, LS 20x washed DawaPlus® 2.0 outperformed the new 20x washed Tsara® Soft: 83.28% [76.48−90.08] vs 59.87% [49.89−69.85], OR 4.07 [2.60−6.36], p < 0.0001.

(a) % KD60 (b) WHO cone assay % 24-hour mortality (c) Tunnel test % 24-hour mortality (d) % blood-feeding inhibition. In all graphs the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition.

(a) % KD60 (b) WHO cone assay % 24-hour mortality (c) Tunnel test % 24-hour mortality (d) % blood-feeding inhibition. In all graphs the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition.

Table 3

I-ACT results against susceptible Anopheles gambiae s.s and resistant An. arabiensis

Test system	Test items	%24-HRS Mortality* [95% CI]	Odds of dying [95% CI]	P-value	% Feeding Inhibition [95% CI]	Odds of Feeding [95% CI]	P-value
Susceptible Anopheles gambiae s.s. (Ifakara strain)	LSª Olyset® unwashed	99.30 [98.25–100.0]	1.00	0.024	94.00 [92.76–99.11]	1.00	0.610
	New Olyset® unwashed	96.28 [93.64–98.93]	0.17 [0.04–0.79]	0.775	97.27 [94.84–99.69]	0.54 [0.05–5.80]	0.693
	LS Olyset® washed	85.73 [76.58–94.86]	1.00	-	91.40 [88.66–94.14]	1.00	0.231
	New Olyset® washed	85.44 [74.29–96.59]	1.09 [0.61–1.93]	-	92.29 [88.73–95.86]	0.84 [0.35–1.99]	0.0001
	Old DawaPlus® 2.0 unwashed	99.66 [98.88–100.0]	-		96.12 [94.40–97.83]	1.00	
	New DawaPlus® 2.0 unwashed	99.65 [98.88–100.0]	-		89.37 [82.90–95.84]	2.14 [0.62–7.47]	
	Old DawaPlus® 2.0 washed	100	-		95.62 [92.81–98.42]	1.00	
	New DawaPlus® 2.0 washed	96.94 [95.56–98.32]	-		83.81 [78.98–88.64]	4.37 [2.67–7.15]	

N = 30 mosquitoes released per strain per test; *Arithmetic mean control-corrected 24-hour mortality with 95% confidence intervals (CI) and Arithmetic mean blood-feeding inhibition with 95% confidence intervals (CI); ªLS = long storage

Test system	Test items	%24-HRS Mortality* [95% CI]	Odds of dying [95% CI]	P-value	% Feeding Inhibition [95% CI]	Odds of Feeding [95% CI]	P-value
Resistant Anopheles arabiensis (Kingani strain)	Old Olyset® unwashed	63.40 [47.83–78.97]	1.00	0.0001	92.10 [88.24–95.95]	1.00	0.213
	New Olyset® unwashed	50.31 [33.42–67.19]	0.49 [0.33–0.72]	0.401	95.16 [91.07–99.26]	0.37 [0.08–1.76]	0.329
	Old Olyset® washed	33.34 [17.91–48.77]	1.00	0.364	84.25 [79.51–88.99]	1.00	0.0001
	New Olyset® washed	37.85 [20.11–55.59]	1.18 [0.81–1.72]	0.393	86.88 [80.34–93.43]	0.67 [0.29–1.51]	0.0001
	Old DawaPlus® 2.0 unwashed	71.30 [56.28–86.32]	1.00		91.57 [88.72–94.41]	1.00	
	New DawaPlus® 2.0 unwashed	68.91 [50.64–87.19]	0.82 [0.53–1.3]		81.78 [75.49–88.07]	2.55 [1.61–4.06]	
	Old DawaPlus® 2.0 washed	48.73 [34.18–63.28]	1.00		83.28 [76.48–90.08]	1.00	
	New DawaPlus® 2.0 washed	45.74 [27.90–63.57]	0.86 [0.60–1.22]		59.87 [49.89–69.85]	4.07 [2.60–6.36]	

N = 30 mosquitoes released per strain per test; *Arithmetic mean control-corrected 24-hour mortality with 95% confidence intervals (CI) and Arithmetic mean blood-feeding inhibition with 95% confidence intervals (CI); [a]LS = long storage

Discussion

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

It was necessary to keep the investigational LLINs under ideal temperature and humidity conditions, as it is known that high temperature may inactivate the insecticide or binder [19, 26]. Proper storage should also avoid direct sunlight as pyrethroids are decomposed by UV light and heat [27]. Several studies have been conducted to evaluate the storage conditions of LLINs. For example, the study conducted in Turkey by Karakus et al. (2016), reported that nets exposed to direct sunlight for six months, had lower efficacy (44.4% 24-hr mortality), than other groups of nets which were not exposed to sunlight (100% 24-hr

mortality) [19]. Atieli et al (2010), showed that drying methods used after washing nets, resulted in significant impact on the efficacy of pyrethroid nets: nets washed 20 times and dried under the shade retained more pyrethroid insecticide (62.5%) than nets directly dried under the sunlight (58.8%) [20]. Furthermore, Peck et al. (2014) reported that the insecticidal activity of the pyrethroid Lambda-cyhalothrin was reduced after 10 weeks of exposure to direct sunlight [26].

LLINs are designed to withstand the high temperatures that may be encountered in the tropics and the findings from this study suggest that nets can retain bio-efficacy for up to five years if stored out of sunlight at the range of 25°C to 33.4°C and 40% – 100% relative humidity. The storage conditions used in this study aligned with the manufacturer specification and WHO guidelines [27, 28]. It should be noted that the LLIN store used for this study was a shipping container (Fig. 4) that uses only passive cooling for the majority of the year. The container is raised above the ground and is situated under a second shade roof to reduce the radiant transfer of heat. It is also equipped with ventilation gaps (similar to the eaves of African houses) to allow air movement through the store. Electric ceiling fans are used only at the hottest times of the year irrespective of the temperature. Therefore, investment in similar storage facility of that of the Ifakara Health Institute for LLINs can ensure longevity efficacy of LLINs at a low running cost.

National malaria programmes should be well informed on the appropriate long-term storage conditions for pyrethroid nets in order for the LLINs to retain their bio-efficacy, if nets are to be stored for extended period before distribution.

The performance of long storage (LS) LLINs varied between net brands and washes in the WHO cone bioassay. DawaPlus® 2.0 LLIN, met the WHO criteria in the standard WHO cone assay without the need to conduct a WHO tunnel test, while Olyset® LLIN failed to meet the criteria based on the cone assay but passed based on WHO tunnel test (Table 2) due to the irritant action of the permethrin insecticide incorporated in Olyset® [22, 29]. This mode of action reduces the probability of mosquito dying from exposure to the insecticide following multiple contacts with net, but also gives Olyset® its feeding inhibition properties that were observed in the I-ACT, allowing protection of human volunteers sleeping beneath them even after 5 years and 2 months of storage. Similar results were observed by Massue et al. (2019) [22]. It was again observed, by Jaramillo et al. (2011), on which permethrin treated net (Olyset® LLIN) reduced contacts of An. albimanus to net surface in the cone test [30].

Both LS Olyset® and DawaPlus® 2.0 failed to meet the WHO mortality efficacy criteria (≥ 80%) against the resistant An. arabiensis (Kingani strain), but the nets still performed well on the feeding inhibition end points. As the candidate LLINs utilize pyrethroid insecticides, it is expected the nets to show reduced efficacy against pyrethroid resistant populations, however, it is clear that LLIN performance was not significantly impaired as a result of long storage, but due to ability of the resistant strain to detoxify pyrethroids [31 – 33]. It is for this reason that piperonyl butoxide-treated insecticidal nets (PBO) nets have been developed [34], PBO is a synergist biochemical substance, combined with pyrethroid, that hinder enzymatic responses of insects against detoxifying pyrethroid for its survival, and allow the pyrethroids

insecticide to finally kills pyrethroid resistant mosquitoes [35]. It is planned to conduct further studies to investigate the long-term storage stability of nets treated with PBO in the future using the set up described here. Although, it is interesting that both nets still performed well on the feeding inhibition end point, which means that long stored pyrethroids LN can still confer protection, therefore reiterate the usefulness in the continuous control of mosquitoes.

Results from the I-ACT with volunteers sleeping beneath the LLINs complemented the evidence provided by the WHO cone assays and allowed for comparison between new nets and long storage nets of the same brand and washing status. Using WHO pass/fail thresholds, findings from WHO cone assays and the I-ACT with LS nets agreed between net brands and washes. Although, using the WHO criteria, both LS nets and new nets passed with the susceptible strain but inconsistent with the resistant strain in the IACT. I-ACT demonstrated higher feeding inhibition and mortality (Table 3, Table 2).. The increased performance of LLINs in the I-ACT might be due to extended exposure time that increased number of contacts between mosquitoes and the LLIN, use of a whole net and the use of a preferred (human) bait by mosquito. Similar I-ACT results have also been observed by Massue et al [22]. However, it should be understood from our findings that, long storage nets performed similarly to the new nets in the I-ACT on both mortality and feeding inhibition.

Study Limitations

The study was conducted as per protocol and WHO guidelines for LLIN evaluations. However, the I-ACT study was not sufficiently powered (< 80%) and one net per condition was used, which limited the study to adequately measure inter-net heterogeneity due to limited number of test nets in the facility. The bursting strength of nets was not evaluated and this needs to be considered to understand the effect of storage on the fabric strength. Therefore, the findings of the study should be cautiously interpreted and we recommend further studies to be conducted in multiple sites with sufficient power to detect differences between nets for each condition, and additional evaluations of bursting strength after storage.

Conclusion

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

Declarations

Ethical approval and volunteer protection

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

Consent for publication

Not applicable

Availability of data and materials

All data are available at Ifakara Health Institute archive

Competing interests

The authors conduct evaluations of vector control products for Ifakara Health Institute.

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Author's contribution

JJM conducted the study, performed data analysis and wrote the first draft of the manuscript; SJM devised study design and critically revised the manuscript; JM performed data entry and cleaning; EMM and DEK performed data collection; EKM and JKS provided critical review of the manuscript; OGO supervised data analysis, supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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References

1. Pryce J, Richardson M, Lengeler C. Insecticide-treated nets for preventing malaria (Review). 2018 2. Kleinschmidt I, Bradley J, Knox TB, Mnzava AP, Kafy HT, Mbogo C, et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. Lancet Infect Dis. 2018. 3. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 2015;526:207–11. 4. Kilian A, Koenker H, Paintain L. Estimating population access to insecticide-treated nets from administrative data: Correction factor is needed. Malar J. 2013;12:1–10. 5. Khanam F, Hossain MB, Chowdhury TR, Rahman MS, Kabir M, Naher S, et al. Exploring the gap between coverage, access, and utilization of long-lasting insecticide-treated nets (LLINs) among the households of malaria endemic districts in Bangladesh. Malar J. BioMed Central; 2018;17:1–12. https://doi.org/10.1186/s12936-018-2610-0 6. Kilian A, Woods Schnurr L, Matova T, Selby RA, Lokko K, Blaufuss S, et al. Evaluation of a continuous community-based ITN distribution pilot in Lainya County, South Sudan 2012-2013. Malar J. BioMed Central; 2017;16:1–13. 7. Krezanoski PJ. Delivering insecticide-treated nets for malaria prevention : innovative strategies. Dovepress J. 2016;39–47. 8. WHO. Guidelines for laboratory and fiel-testing of long-lasting insecticidal nets. 2013; 9. WHO. List of WHO Prequalified Vector Control Products. 2018;1–5. https://www.who.int/pq-vector-control/prequalified-lists/PQT_VC_17July2018.pdf?ua=1 10. WHO. Guidelines for malaria vector control. 2019. 11. WHO. Estimating population access to ITNs versus quantifying for procurement for mass campaigns. 2014;2013–4. 12. Koenker H, Arnold F, Ba F, Cisse M, Diouf L, Eckert E, et al. Assessing

whether universal coverage with insecticide-treated nets has been achieved: Is the right indicator being used? Malar J. BioMed Central; 2018;17:1−11. https://doi.org/10.1186/s12936-018-2505-0 13. WHO/GMP. Achieving and maintaining universal coverage with long-lasting insecticidal nets for malaria control. Who [Internet]. 2017;4. http://www.who.int/malaria/publications/atoz/who_recommendation_coverage_llin/en/ 14. AMP. 4: LLIN procurement and pipeline monitoring. 2017;1−14. 15. William R Brieger. The challenge of using and misusing insecticide-treated bed nets. Afr Health. 2017;13−5. 16. WHO. World Malaria Report [Internet]. 2018. www.who.int/malaria 17. Koenker H. EClinicalMedicine More is More: Are We Delivering Enough LLINs? EClinicalMedicine. Elsevier Ltd; 2018;1:5−6. https://doi.org/10.1016/j.eclinm.2018.07.005 18. Gomes de Mattos R, Oliveira F, Leiras A, Baptista de Paula Filho A, Gonçalves P. Robust optimization of the insecticide-treated bed nets procurement and distribution planning under uncertainty for malaria prevention and control. Ann Oper Res. Springer US; 2018; https://doi.org/10.1007/s10479-018-3015-8 19. Karakuş M, Kasap ÖE, Günay F, Oğuz G, Demir S, Suner A, et al. Effects of environmental factors and storage conditions on the performance of Olyset Plus against sand flies in WHO cone bioassays. Trans R Soc Trop Med Hyg. 2016;110:252−7. 20. Atieli FK, Munga SO, Ofulla A V., Vulule JM. Wash durability and optimal drying regimen of four brands of long-lasting insecticide-treated nets after repeated washing under tropical conditions. Malar J. 2010;9:1−10. 21. USAID. Long-Lasting Insecticide-Treated Bed Net Packaging Considerations. 2014;9.http://deliver.jsi.com/dlvr_content/resources/allpubs/logisticsbriefs/LLINPackCons.pdf 22. Massue DJ, Lorenz LM, Moore JD, Ntabaliba WS, Ackerman S, Mboma ZM, et al. Comparing the new Ifakara Ambient Chamber Test with WHO cone and tunnel tests for bioefficacy and non - inferiority testing of insecticide - treated nets. Malar J. BioMed Central; 2019;1−15. 23. WHO. Report of the Thirteenth Whopes Working Group Meeting. World Heal Organ. 2009;5:1−73. 24. Kaufmann C. Methods in Anopheles Research. Mosq News. 2014;4th Editio:343. 25. Shi P, Tsai CL. Regression model selection - A residual likelihood approach. J R Stat Soc Ser B Stat Methodol. 2002;64:237−52. 26. Peck GW, Ferguson HJ, Lepage JT, Hebert VR, O'Neal SD, Walsh DB. Evaluation of sunlight-exposed pyrethroid-treated netting for the control of face fly and housefly (Diptera: Muscidae). Pest Manag Sci. 2014;70:123−9. 27. WHO. Who Specifications and Evaluations for Public Health Pesticides Deltamethrin Long-Lasting. 2015;1−96. 28. WHO. Who Specifications and Evaluations for Public Health Pesticides Permethrin- Incorporated into filaments. 2014;1−21. 29. Rafinejad J, Vatandoost H, Nikpoor F, Abai MR, Shaeghi M, Duchen S, et al. Effect of washing on the bioefficacy of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) against main malaria vector Anopheles stephensi by three bioassay methods. J Vector Borne Dis. 2008. 30. Jaramillo GI, Robledo PC, Mina NJ, Muñoz JA, Ocampo CB. Comparison of the efficacy of long-lasting insecticidal nets under laboratory conditions. Mem Inst Oswaldo Cruz. 2011;106:606−12. 31. Kisinza WN, Nkya TE, Kabula B, Overgaard HJ, Massue DJ, Mageni Z, et al. Multiple insecticide resistance in Anopheles gambiae from Tanzania: A major concern for malaria vector control. Malar J. BioMed Central; 2017;16:1−10. 32. WHO/ GMP. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes: Second edition. World Heal Organ Tech Rep Ser. 2016;22. https://www.who.int/malaria/publications/atoz/9789241511575/en/ 33. Alemayehu E, Asale A, Eba K, Getahun K, Tushune K, Bryon A, et al. Mapping insecticide resistance and characterization of

resistance mechanisms in Anopheles arabiensis (Diptera: Culicidae) in Ethiopia. Parasites and Vectors. 2017;10:1–11. 34. Protopopoff N, Mosha JF, Lukole E, Charlwood JD, Wright A, Mwalimu CD, et al. Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two fact. Lancet Open Access article under the CC BY 4.0 license; 2018;391:1577–88. http://dx.doi.org/10.1016/S0140-6736(18)30427-6 35. Gleave K, Lissenden N, Richardson M, Choi L, Ranson H. treated nets to prevent malaria in Africa (Review). 2018;

Figures
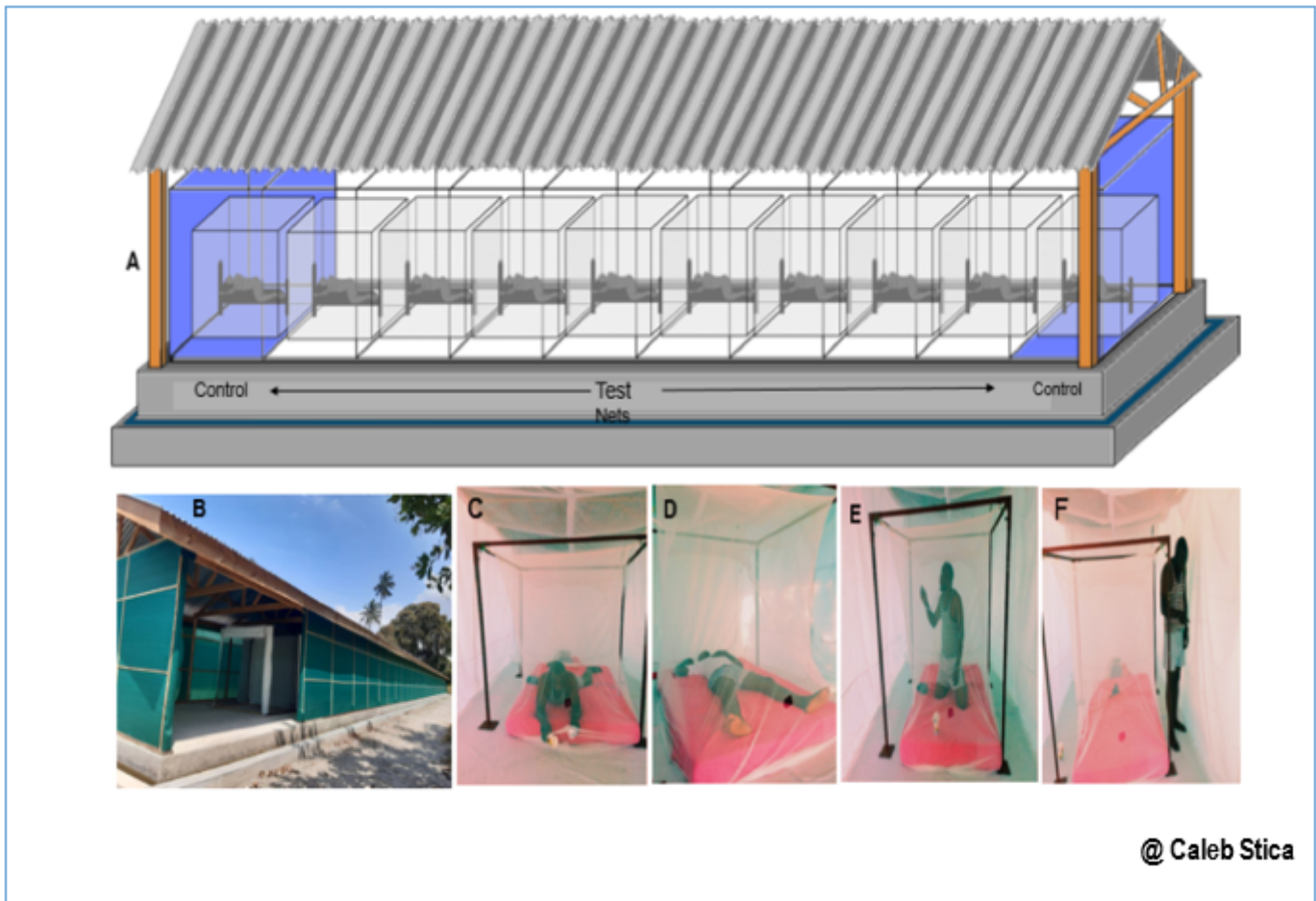
Figure 1

I-ACT Assay A- Schematic diagram of the Ifakara Ambient Chamber Test (I-ACT with10 chambers); B- I-ACT at IHI-Bagamoyo branch; C- Sleeper releasing mosquitoes within chamber outside the net; D – volunteer (sleeper) sleeping in side net within a chamber; E- Sleeper collecting mosquitoes using mouth aspirator (siphon) inside net within chamber, F- Sleeper collecting mosquitoes using siphon outside net within chamber.
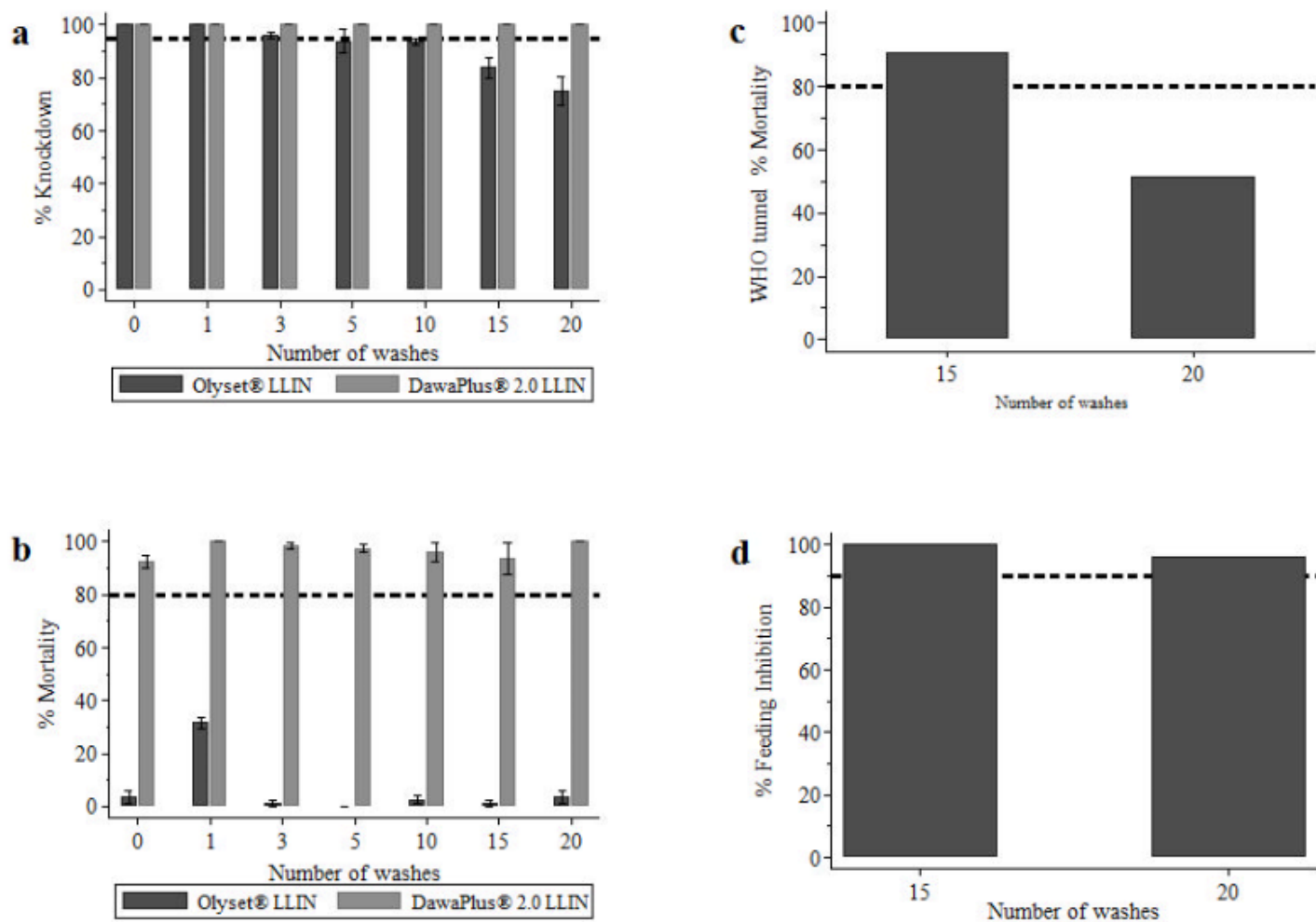
Figure 2

WHO bio-assay results against susceptible An. gambiae s.s (Ifakara strain) (a) % KD60 (b) WHO cone assay % 24-hour mortality (c) Tunnel test % 24-hour mortality (d) % blood-feeding inhibition. In all graphs the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition.
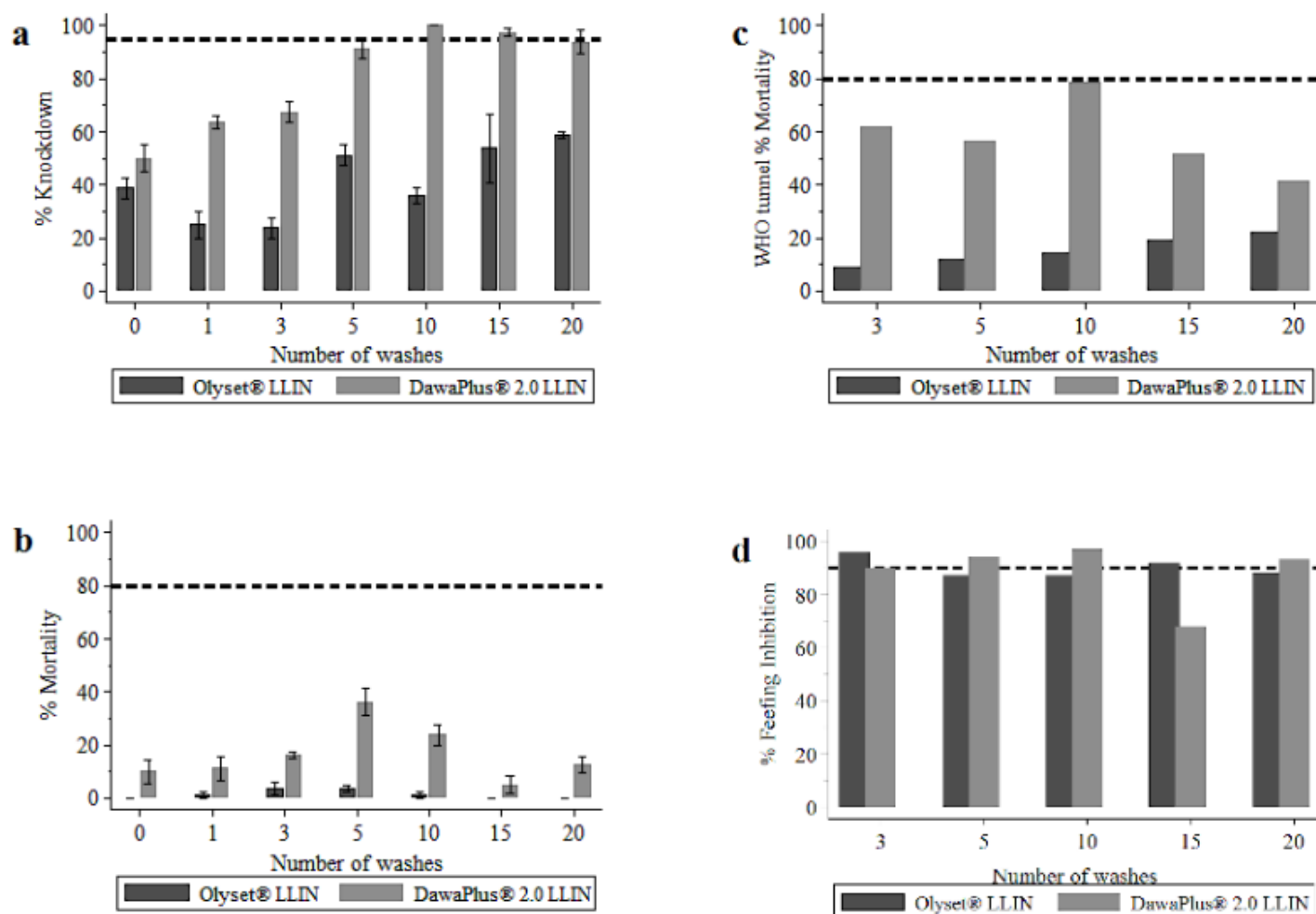
Figure 3

WHO bio-assay results against resistant An. arabiensis (Kingani strain) (a) % KD60 (b) WHO cone assay % 24-hour mortality (c) Tunnel test % 24-hour mortality (d) % blood-feeding inhibition. In all graphs the dashed line is the WHO cut off criteria, 95% for KD60, 80% for mortality, and 90% for blood-feeding inhibition.

Figure 4

The Bagamoyo IHI LLIN storage facility