Optimizing and Refining Net Washing Methodologies: A Comprehensive Analysis of Current Practices and Recommendations for Future

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Acronym List

AI	Active Ingredient
CIPAC	Collaborative International Pesticides Analytical Council
HPLC	High Performance Liquid Chromatography
KD	Knockdown
LLIN	Long Lasting Insecticidal Net
PQ	Pre-Qualification
RPM	Rotations Per Minute
VCT	Vector Control Team
WHO	World Health Organisation
WHOPES	World Health Organisation Pesticide Evaluation Scheme

Introduction

The World Health Organization (WHO) fervently supports the advancement of innovative public health pesticides, with a particular emphasis on long-lasting insecticidal nets (LLINs) that utilize non-pyrethroid insecticides, insecticide mixtures, and combination LLINs containing synergists or repellents. These pioneering LLINs are designed to tackle the issues of insecticide resistance within mosquito populations. To ensure their efficacy and safety, all LLINs, regardless of composition, must undergo rigorous evaluation via laboratory and experimental hut studies. These assessments should establish non-inferiority or additional advantages over traditional pyrethroid-treated LLINs in terms of mosquito mortality, blood-feeding inhibition, and personal protection. Furthermore, the safety of these products must not be jeopardized when employing mixtures or supplementary agents [1].

To gain WHO PQ/VCT approval for long-lasting insecticidal mosquito nets, a comprehensive dossier containing quality data is required. This data is obtained through analytical testing, which determines suitable wash intervals for artificial aging and plots wash resistance curves. Subsequently, bioassays are employed to measure insecticide surface content effectiveness and evaluate the net's ability to maintain biological activity after twenty standard washes. However, the correlation between bioassay results and a net's physiochemical properties before and after washing remains unclear.

There are several sets of WHO guidelines detailing net washing procedures within these multiple washing protocols have been developed and recommended by the WHO, though they were devised independently, resulting in methodological variations that make their comparability ambiguous. The primary methods include those detailed for use in WHOPES Phase I laboratory studies, WHOPES Phase II and III small-scale field trials (semi-field/experimental hut) and large-scale field trials (randomized control trial or cluster randomized control trial), as well as the CIPAC washing procedure, which offers complementary chemical analysis for WHOPES Phase I, II, or III testing.

Aims and objectives

- Review of recommended methodologies for net washing.
- Review of published literature for net washing details.

Background

Comparison between wash methods

The first washing method used for evaluating the effectiveness of long-lasting insecticidal mosquito nets is called the WHO Phase 1 wash testing. This protocol is conducted in a laboratory setting and involves testing small pieces of the net to evaluate the regeneration of insecticidal activity, efficacy, and wash-resistance. The purpose of washing in this protocol is to deplete the surface AI content to 0 (or below a detectable limit) to allow for regeneration following washing, which demonstrates the efficacy and wash-resistance of the net against a fully susceptible Anopheles species after washing.

The endpoints measured in this method are regeneration and efficacy. Regeneration refers to the time required for insecticidal regeneration of the net after washing to prewash levels or a target dose which still gives biological activity (prewash doses may be higher than target dose due to blooming for some nets). The depletion of insecticide and return to prewash levels is primarily assessed by cone bioassay, although HPLC chemical testing may also be used as a supplementary method alongside bioassay data. Efficacy is demonstrated by achieving either ≥ 80% mortality or ≥ 95% knockdown in a cone bioassay for nets washed twenty times. If the twenty wash bioassay data fail to show efficacy by cone test, then tunnel test data may be used. Nets washed at least twenty times that meet the criteria of the tunnel test (≥ 80% mortality or ≥ 90% blood-feeding inhibition) are deemed effective and undergo phase II testing. Additionally, it should be noted that the knockdown and mortality thresholds are not equivalent (95% KD ≈ 20-30% mortality) for several standard pyrethroid nets either prior to, or across the washing cycles. [2]

Phase II wash testing is a small-scale semi-field trial that involves evaluating whole nets in experimental huts. In this protocol, wash resistance and efficacy of an unwashed net are assessed by comparing washed and unwashed nets with a positive control reference net that is also washed and unwashed. The study includes an untreated net made of the same or similar material as the candidate net, a polyester net, a reference LLIN both unwashed and washed twenty times, and a candidate LLIN both unwashed and washed twenty times. This testing should be informed by the wash resistance profile identified in Phase I bioassay and ideally with concurrent CIPAC analysis. It is not clear how Phase I and CIPAC results correlate with results from Phase II wash resistance and efficacy testing.

The primary outcomes for this testing are deterrence, exophily, blood feeding inhibition, and both immediate and delayed mortality. Deterrence is the reduction in hut entry compared to untreated control huts, and it is measured by calculating the personal protection of the net using the formula:

$$Personal\ protection\ (\%) = 100\ \times \frac{(B_u - B_t)}{B_u}$$

Here, B_u is the total number of mosquitoes that have blood-fed in the huts with untreated nets, and B_t is the total number of mosquitoes that have blood-fed in the huts with treated nets. Exophily is the proportion of mosquitoes found in the exit and veranda traps. Blood feeding inhibition is the reduction in blood feeding compared to untreated control huts.

Immediate and delayed mortality are the proportions of mosquitoes entering the huts that are found dead in the morning (immediate) or after being caught alive and held for 24 hours with access to sugar (delayed). This is measured by calculating the killing effect using the formula:

$$Killing\ effect\ (\%) = 100\ \times \frac{(K_t - K_u)}{T_u}$$

Where K_t is the number of mosquitoes killed in the huts with treated nets, K_u is the number of mosquitoes killed in the huts with untreated nets, and T_u is the total number of mosquitoes collected from the huts with untreated nets.

To complement the previous methods, CIPAC testing is used to determine the wash resistance index of net pieces through chemical analysis by HPLC. This method is comparable to Phase I testing and is often done alongside it and other phases of testing. The wash resistance index is calculated using the total active ingredient content (in g/kg) after four washing cycles (t_4) and before washing (t_0) according to the formula:

$$w = 100 \times \sqrt[4]{(t_4/t_0}$$

This provides a percentage measure of the wash resistance index [3].

See the following table for a full summary and comparison of these wash methods:

Table 1. Main parameters assessed in phase I, II and III studies of long-lasting insecticidal mosquito nets.

Phase	Type of Study	Parameters Measured	Purpose of washing	Endpoint measured
I	Laboratory (net pieces)	• Regeneration of insecticidal activity	Depletion of surface AI content to allow for regeneration following washing.	• Regeneration ○ The time required for insecticidal regeneration of the LLIN after washing to prewash levels/target dose.

		• Efficacy and wash-resistance	Efficacy and wash-resistance of the LLIN against a fully susceptible Anopheles species.	○ Assessed by cone bioassay. • Efficacy ○ Nets washed at least twenty times that meet the criteria of WHO cone bioassays (≥ 80% mortality or ≥ 95% knockdown) ○ If twenty wash cone bioassay data do not show efficacy by cone test, then tunnel test data may be used. ○ Nets washed at least twenty times that meet the criteria of tunnel test (≥ 80% mortality or ≥ 90% blood-feeding inhibition) ○ Nets that meet these efficacy criteria undergo phase II testing
II	Small-scale field trial (whole nets)	• Wash-resistance • Efficacy as measured by vector mortality and blood-feeding inhibition	Comparison of washed and unwashed nets in comparison with a washed and unwashed positive control reference net.	Study Arms: • Untreated net of the same or similar material or a polyester net. • Reference LLIN unwashed. • Reference LLIN washed twenty times. • Candidate LLIN unwashed. • Candidate LLIN washed twenty times. Primary outcomes: • Deterrence: the reduction in hut entry relative to untreated control huts. ○ $Personal\ protection\ (\%) = 100 \times \frac{(B_u - B_t)}{B_u}$ ○ Where B_u is the total number blood fed mosquitoes in the huts with untreated nets and B_t is the total number of blood fed mosquitoes in the huts with treated nets. • Exophily: the proportion of mosquitoes found in the exit and veranda traps.

				• Blood feeding inhibition: the reduction in blood feeding in comparison with untreated control huts. • Immediate and delayed mortality: the proportions of mosquitoes entering the huts that are found dead in the morning (immediate) or after being caught alive and held for 24 hours with access to sugar (delayed). ○ $Killing\ effect\ (\%) = 100 \times \frac{(K_t - K_u)}{T_u}$ ○ Where K_t is the number of mosquitoes killed in the huts with treated nets, K_u is the number of mosquitoes killed in the huts with untreated nets, and T_u is the total number of mosquitoes collected from the huts with untreated nets.
III	Large-scale field trial	• Long-lasting insecticidal efficacy • Rate of loss or attrition of nets • Physical durability of netting material • Community acceptance • Safety	Questions 3.14 to 3.18 of the "Sample questionnaire for monitoring durability of nets in phase III studies" relate to net washing behaviour:	• Has the net ever been washed? • When was the last time you washed the net? • What type of soap was used? • How long did the net soak for? • Was the net scrubbed hard or beaten on a hard surface (e.g., rocks, with sticks. • Where was the net dried?
CIPAC		• Wash resistance index • Performed on net pieces so comparable to Phase I	Determination of wash resistance index	$w = 100 \times \sqrt[4]{(t_4/t_0}$ Where: w = wash resistance index expressed as a percentage; t_4= total active ingredient content (in g/kg) after 4 washing cycles; and t_0= total active ingredient content (in g/kg) before washing.

Methods

Guideline Review

WHO Guidelines

There are several sets of WHO (World Health Organization) guidelines containing details of net washing methodologies. To better understand the net washing methodology these documents were reviewed for methodological details to identify areas of misinterpretation as well as identifying any potential knowledge gaps.

CIPAC Guidelines

Additionally, a presentation detailing the development of the CIPAC methodology was assessed alongside the CIPAC guidelines.

Literature review

To investigate the use of the WHO cone bioassay for the efficacy evaluation of unused pyrethroid LLINs, Mbwambo et al. (2022) conducted a literature review of LLIN efficacy studies, durability studies, or WHOPES specification reports published between 2001 and 2021 [4]. The search was conducted in October 2021 on PubMed and PubMed Central, , using the keywords "bio-efficacy" or "cone bioassay tests" and "tunnel tests" or "insecticide treated nets" and "long-lasting insecticidal nets" and on Google Scholar using the search terms "WHOPES working group meeting". They used strict inclusion criteria to identify reports using standard WHO evaluation methods on unused pyrethroid LLINs with Anopheles mosquitoes that reported both KD60 and M24. Their search identified 2,362 titles, which they screened to identify relevant papers. They fully screened seventy publications and included sixty in their final selection.

These papers identified by Mbwambo were then taken analysed for their reporting of the bioassay methodology and referencing for this method.

Data extracted from selected publications included ITN type (brand name, active ingredient, manufacturing technology, manufacturing date or year, batch/lot number), bioassay results (mainly KD60 and M24), the Anopheles strain used in the bioassays and where and when the study was conducted. From this list of papers those that included net washing as part of their methods were extracted and assessed for methodological details.

An additional search was conducted in March 2023 on PubMed and PubMed Central, , using the keywords "bio-efficacy" or "cone bioassay tests" and "tunnel tests" or "insecticide treated nets" and

"long-lasting insecticidal nets" restricting the date range to October 2021 to March 2023 to identify any additional papers of relevance that had been published after the Mbwambo 2022 study. No additional papers were identified which fit inclusion criteria outline above.

Results

Guideline review

In reviewing the guidelines for net washing methodologies, several variations have emerged over time. The 2005 Guidelines [5] outlined the procedures for Phase 1 and Phase 2. In Phase 1, regeneration time and standard washing were assessed, with samples held at 30 degrees Celsius. The washing procedure involved shaking net samples in deionised water containing Savon de Marseilles soap flakes at a specific concentration. The samples were then washed and dried one or three times, with regeneration curves established for both scenarios. The longer regeneration time was chosen, and testing was performed at various intervals post-wash. Standard WHO cones were used for bioassays with pooled mosquito samples. In Phase 2, a 10L water wash was conducted for ten minutes at twenty RPM.

The 2006 Guidelines [6] maintained the same Phase 1 procedure as the 2005 Guidelines, while Phase 2 involved a 10 L wash with Savon de Marseilles soap, agitated for six minutes within a total period of 10 minutes at a rate of twenty RPM.

Finally, the 2013 Guidelines [7] provided more detailed instructions for both phases. In Phase 1, four nets were required, and fourteen net pieces were collected from each net. Eight of those pieces were used for the regeneration study, while twenty-eight pieces were used for wash resistance testing at various intervals. An additional twenty pieces were stored for chemical analysis. Phase 2 remained the same as in the previous guidelines.

There are also several proposed schemes for sampling nets during various phases of testing (refer to Figures 1, 2, and 3). As discussed later in the literature review, the storage conditions for nets and net samples exhibit significant variation. Considering this, harmonization of the sampling procedures and clearer instructions for sample storage between distinct stages of a study are essential.

Figure 1 - Net sampling protocol used in during CIPAC wash method development. Figure is from WHO 2013 "Recommended positions from which netting pieces should be taken" for phase II studies.

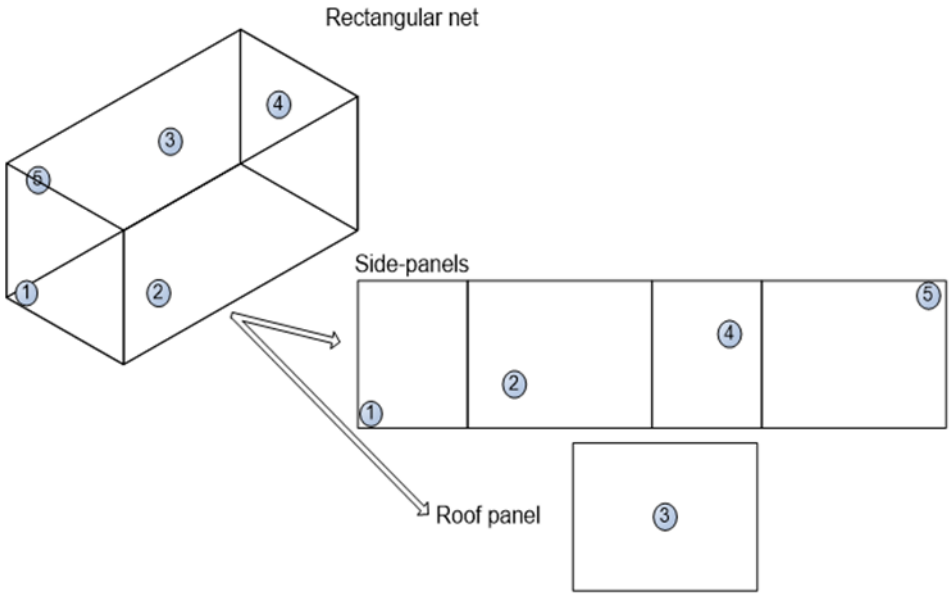


Figure 2 - Sampling scheme for fourteen pieces of netting from each net, including positions HP1–HP5 for chemical assay for Phase I studies.

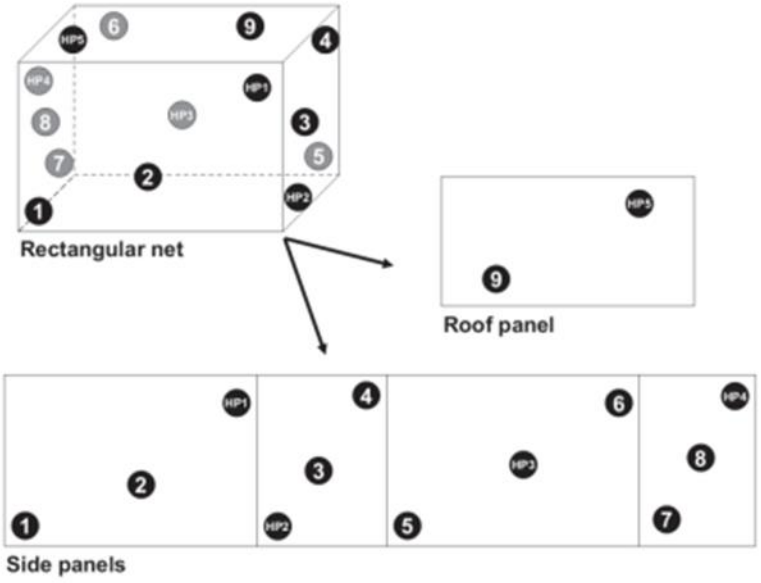


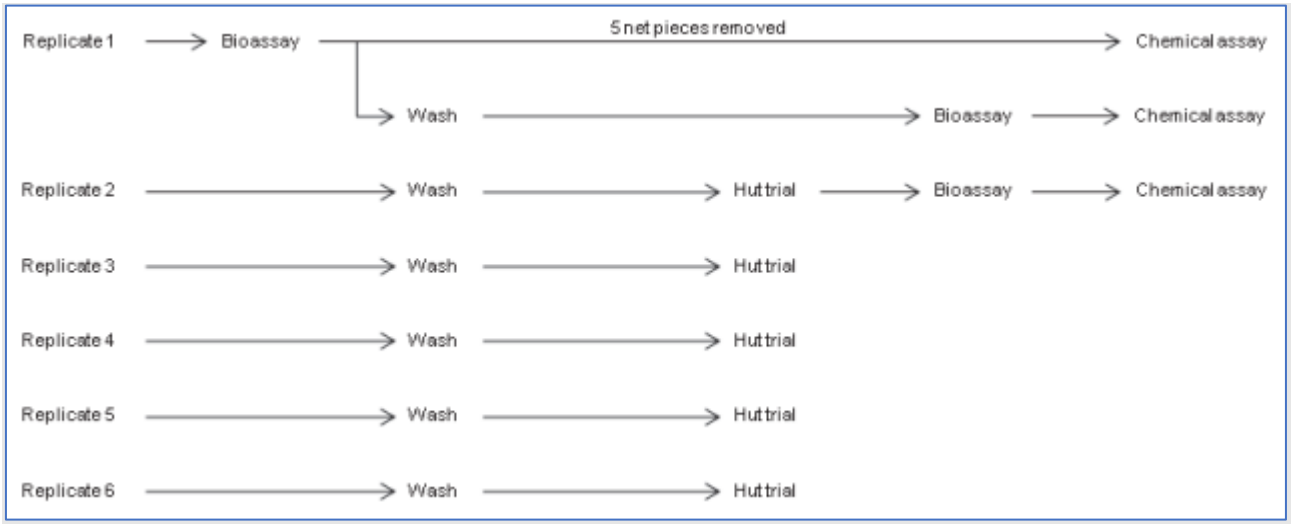
Figure 3 - Scheme for washing of nets to determine wash resistance and insecticide retention rates during phase I.

Net pieces[a]	Number of standard washes																									
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
4	Efficacy of unwashed nets tested[b]	Washed once, efficacy tested and net pieces stored at 4 ^{0}C for chemical analysis																								
4	Washed 3 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									
4	Washed 5 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									
4	Washed 10 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									
4	Washed 15 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									
4	Washed 20 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									
4	Washed 25 times, efficacy tested and pieces stored at 4 ^{0}C for chemical analysis																									

[a] One piece is selected randomly from each of the four nets

[b] These unwashed pieces are not used for chemical assay; see section 1.1.1 for sampling plan for chemical assay of unwashed pieces

Figure 4 - Washing, bioassay and chemical assay of each of six net replicates for candidate and reference long lasting insecticidal nets in an experimental hut trial (phase II)



CIPAC methodology

The CIPAC wash method involves creating a stock solution by mixing 80 ml of water, 12 g of sodium oleate, and 8 g of polyoxyethyleneglycolmonostearate and heating it to 50 degrees Celsius. To wash the net pieces, 2.5 mL of the stock solution is combined with 500 mL of deionised water, and 25x25cm net pieces are added. The samples are mixed by inverting them 10 times before leaving them undisturbed in a water bath or oven maintained at 30°C ± 2°C for 10 minutes. After washing, the net pieces are rinsed twice in deionised water at 30°C ± 2°C, with each rinse involving 10 inversions and a 10-minute rest period. [7]

Post-rinsing, the net samples are folded, placed in a bottle, and stored at 40°C ± 2°C for 22 hours ± 2 hours. This process is repeated three more times for a total of four washes to fully deplete the insecticides. The CIPAC washing agent can be used for up to four weeks if it is kept sealed, stored in the dark, and maintained at 4°C.

The CIPAC method was developed to provide a chemical analysis of insecticide content of nets that is comparable to the WHO washing protocols but with a higher throughput. Results are used to decide on an LLIN's specifications, and is part of the former JMPS, now WHO PQ/VCT, set of specifications. During the development process, Marseille soap at 2 g/L (which is the WHO recommended detergent and concentration for Phase I and II testing) and CIPAC washing agents at concentrations of 2 g/L, 4 g/L, and 8 g/L were compared. A range of pyrethroid-only nets of several types were washed with these detergents and then analysed using the appropriate CIPAC method for the net type. The nets assessed included Olyset® (permethrin in incorporated nets), DuraNet® (alpha-cypermethrin in incorporated nets), PermaNet® 2.0 (deltamethrin in coated nets), and NetProtect® (deltamethrin in incorporated nets).

This CIPAC method development revealed a reasonable level of agreement between the CIPAC wash method and the WHO Phase I wash method at a concentration of 4 g/L of CIPAC washing agent. However, no agreement has been demonstrated side by side for phase I and II testing or for CIPAC and phase II testing for a variety of nets [3].

Multiple studies have shown that household practices vary by country and region and can affect net longevity. These practices, such as the frequency and method of washing and drying nets, have been shown to be different in different regions. For instance, beating nets on rocks or other hard surfaces to wash them is a widespread practice, but it can cause wear of the fabric, which reduces their durability.

Similarly, hanging or drying nets in the sun can cause the depletion of insecticide, as UV can react with insecticides or other ingredients of the net formulation [8]. As a result, questions have been raised about how "field relevant" these wash methods are and whether they produce comparable results to nets aged and washed naturally in the field.

To address these issues, net washing methodologies could be designed to reflect the real-world effects of net washing. This could be accomplished by redeveloping new washing procedures. Due to the potential physical degradation caused by washing, it may also be appropriate to assess bursting strength or other measures of physical durability alongside wash methods.

Phase III testing is a part of the WHO testing pathway that does not include any methods on washing. These studies involve large-scale field trials. Within the questionnaire for monitoring durability of nets in Phase III studies, several questions relating to net washing behaviour are included:

1. Has the net ever been washed?
2. When was the last time you washed the net?
3. What type of soap was used?
4. How long did the net soak for?
5. Was the net scrubbed hard or beaten on a hard surface (e.g., rocks, with sticks.
6. Where was the net dried?

Phase III testing data was only generated under WHOPES for a few nets, and for some of those nets the reported study did not fully conform to WHOPES guidelines. As a result, there has been little publicly available data generated on net washing behaviours through this protocol [1,9].

Literature review

Based on the methodological details extracted from the selected papers, it appears that referencing of guidelines and years out of date vary widely among the studies. Out of the sixty papers selected in the Mbwambo et al. (2022) [4] review, 7% did not reference any guidelines, while 4% referenced published papers for guidelines. Of the papers that did reference guidelines, 11% used the 1998 guidelines, while 37% used the 2005 guidelines. There were no papers that referenced the 2006 guidelines, and only 8% referenced the 2011 guidelines, while 33% referenced the 2013 guidelines. In terms of years out of date of the referenced guidelines, the selected papers varied widely, with 38% using guidelines that were up

to date, while 38% used guidelines that were 8 years out of date. There is a need for greater consistency in the referencing of guidelines and adherence to up-to-date guidelines to ensure the reliability and comparability of results across studies.

Methodological details extracted from the papers also included the sources of the nets used in the studies, which were diverse and mostly included direct sourcing from the chemical company, local markets, homes, research institutes, storage facilities, and the Ministry of Health. However often it was not stated at all where the nets were sourced from. The storage conditions for the nets varied widely, with some studies not stating the storage conditions and others using foil, plastic bags, fridges, or envelopes to store the nets.

The size of the net sample used in the bioassay varied, with some studies using 25 x 25 cm samples, while others used 30 x 30 cm samples or the entire net. The age of the mosquitoes used in the bioassay also varied, with some studies using 2-3 day old mosquitoes, while others used 2-4, 2-5, or 3-5 day old mosquitoes, or did not state the age at all. The number of mosquitoes per cone varied, with some studies using five mosquitoes per cone, while others used 8-10 or 10 mosquitoes per cone, or did not state the number. The negative control netting used in the studies varied, with non-specific "untreated" nets being the most common.

The papers also reported the number of subsamples per net, the number of nets, the sample size, and the cones per subsample. The number of subsamples per net ranged from 1-10, the number of nets ranged from 1-932, and the sample size ranged from 20-20,000 mosquitoes. The cones per subsample ranged from 1-16.

The analysis of methodological details in the selected studies demonstrates a significant variability in referencing guidelines, net sources, storage conditions, sample sizes, and bioassay procedures. The lack of consistency in adhering to up-to-date guidelines and methodological practices poses challenges for the reliability and comparability of results across studies. To ensure accurate and meaningful conclusions in the field of long-lasting insecticidal nets research, it is crucial to promote the use of standardized and updated guidelines, as well as transparency in reporting methodological details. By encouraging the adoption of consistent practices, researchers can more effectively contribute to the body of knowledge and support the development of effective vector control strategies and public health interventions.

From the initial list of papers in Mbwambo et al. (2022) those that included net washing as part of their methods were extracted and assessed for methodological details.

The selected studies employ various washing methods for evaluating long-lasting insecticidal nets (LLINs). Sood et al. (2011) [10] used a 10-minute detergent soak with a pH of 9.0-9.5, followed by rinsing, drying, and storage at room temperature. Rafinejad et al. (2008) [11] followed the WHO 2005 guidelines, washing net samples in deionized water with soap and shaking at a specific rate before drying and storing at 30°C. Clegban et al. (2021) [12] adapted the WHO 2013 guidelines for washing, using well water and soap with manual agitation and specified time intervals. Nets were dried in the shade and stored at ambient temperature. Musa et al. (2020) [13] also adhered to the WHO 2013 guidelines, washing net samples up to 20 times following standard procedures. Finally, Camara et al. (2018) [14] washed nets 20 times as per the standard WHO washing procedure used in phase II trials, with a regeneration time of one day.

The level of detail provided in these studies varies, with some specifying the type of detergent, water, agitation method, and storage conditions, while others only mention the number of washes and guideline adherence. Although most studies followed the WHO guidelines, there were differences in the washing methods, such as the detergent used, water type, and agitation techniques. These variations might impact the consistency and comparability of the results across studies, highlighting the need for harmonized washing procedures and more explicit guidance to ensure accurate evaluation of LLINs.

Recommendations

As we move forward in evaluating and improving net wash methodologies, it is crucial to develop methods that meet specific requirements. These include applicability to both net pieces and whole nets, calculation of regeneration time, assessment of bioefficacy endpoints, calculation of appropriate endpoint metrics for semi-field and field testing, chemical investigation of net samples, and comparability between methods in terms of insecticide depletion from the net. Additionally, these methods should be representative of degradation of nets in the field and complementary to lab, semi-field, and field studies.

To better understand the specific parameters of wash methods and their impact on results, we propose addressing the following questions:

1. Is there a correlation between phase I and II studies for the same nets, and do differences in wash methods play a role?
2. How closely are the guidelines being followed, and is reproducibility of results ensured?
3. How representative are standardized wash methods of net degradation in use?
4. What is the correlation between cone test results or experimental hut results following artificial aging and results conducted during durability monitoring on naturally aged nets?
5. Do we have enough data and appropriate methods to capture usage behaviours?
6. Are we accurately measuring regeneration time?
7. Where do we need more detail, and where is the method vague or subject to interpretation?
8. How important is drying as part of the protocol for multiple washes, and what is the effect of not drying completely between washes and of the drying conditions?
9. How meaningful is the relationship between the measures used for setting specifications and measuring quality, and the efficacy of the nets?

It is important to establish clarity on the purpose of washing in the context of long-lasting insecticidal nets. Are the aims of washing more about quality analysis or performance evaluation. Although these objectives are related, they differ in terms of interpretation, and it is essential to address this distinction.

What we need to establish is a connection between the net specifications and a measure of efficacy. By understanding this relationship, we can ensure that washing protocols serve both quality analysis and performance evaluation purposes effectively.

Conclusion

Multiple net wash methodologies currently exist, but the correlation between these methods in terms of insecticide depletion and subsequent regeneration remains unclear. Each wash method is designed with a specific purpose in mind, but the guidance could benefit from optimization. Moreover, there is variability in how certain aspects of net washing, such as drying, are conducted. The regeneration procedure involves numerous wash and rinse steps, generating a significant amount of waste per net piece (2.5L of wastewater per 25x25cm net piece). It is plausible that experimental work aimed at optimizing and streamlining this method could prove valuable.

Initial work done during the CIPAC method development has demonstrated agreement between Phase I and CIPAC wash methods during the development of the CIPAC methodology. As a result, it is recommended to compare the current Phase I and II wash methods with each other and determine whether they exhibit the same regeneration time by chemical analysis and efficacy by bioassay for a standard pyrethroid-only net. Based on the agreement between the methods, further investigation could explore the impact of wash temperature, interval, agitation, wash time, and drying procedure for these wash methods. Additionally, establishing clear and relevant methods for assessing net washing behaviours is essential to support field trials.

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