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Control of Neglected Tropical Diseases
WHO Pesticide Evaluation Scheme
http://www.who.int/whopes/en

REPORT OF THE THIRTEENTH *WHOPES* WORKING GROUP MEETING

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WHOPES
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WHO/HQ, GENEVA
28—30 JULY 2009

Review of:

OLYSET® LN
DAWAPLUS®2.0 LN
TIANJIN YORKOOL® LN

World Health Organization

WHO/HTM/NTD/WHOPES/2009.5

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ABBREVIATIONS

AI	active ingredient
BF	blood-feeding
CART	Classification and Regression Tree (software)
FIOH	Finnish Institute of Occupational Health
GMP	Global Malaria Programme (WHO)
HPLC	high-performance liquid chromatography
HR	hazard ratio
ITN	insecticide-treated net
KN	knockdown
LIN/IRD	Laboratoire de Lutte contre les Insectes nuisibles, Institut de Recherche pour le Développement, Montpellier, France
LN	long-lasting insecticidal mosquito net
MKDT	median knockdown time
MOH	Ministry of Health
PCR	polymerase chain reaction
RH	relative humidity
RSD	relative standard deviation
SC	suspension concentrate
WHOPES	WHO Pesticide Evaluation Scheme

1. INTRODUCTION

The thirteenth meeting of the WHOPES Working Group, an advisory group to the WHO Pesticide Evaluation Scheme (WHOPES), was convened at WHO headquarters in Geneva, Switzerland, from 28 to 30 July 2009. The objective of the meeting was to review the reports of testing and evaluation of three long-lasting insecticidal mosquito nets (LNs) for prevention and control of malaria: Olyset® (Sumitomo Chemical, Japan); DawaPlus® 2.0 (Tana Netting, Thailand); and Tianjin Yorkool LN (China).

The meeting also reviewed WHOPES recommendations on PermaNet® 3.0 (Vestergaard-Frandsen, Switzerland), in the light of the revised product claims, and made appropriate recommendations (see Annex I).

The meeting was attended by 13 scientists (see Annex II: List of participants). Professor Dr Marc Coosemans was appointed as Chairman and Dr Mark Rowland as Rapporteur. The meeting was convened in plenary and group sessions, in which the reports of the WHOPES supervised trials and relevant published literature and unpublished reports were reviewed and discussed (see Annex III: References). Recommendations on the use of the above-mentioned products were made.

2. REVIEW OF OLYSET®

Olyset® Net is a long-lasting insecticidal mosquito net (LN) manufactured by Sumitomo Chemical, Japan. The product is made of high-density mono-filament polyethylene yarn (weight 50 g/m²), containing technical permethrin 2% (w/w) as active ingredient (AI), corresponding to about 1000 mg of AI/m² or 20 g AI/kg. The insecticide is incorporated into filaments and migrates through the threads of the net by diffusion. Olyset Net is made of wide mesh (4 mm x 4 mm).

Olyset Net received the full recommendation of WHOPES in October 2001[1] for the use in prevention and control of malaria, which was before the current WHO guidelines for testing and evaluation of LNs were published.[2] The recommendation was based on village level studies, including 3- to 4-year trials in West Africa and WHOPES supervised trials at Phase I and II. WHO *interim specifications* for permethrin long-lasting (incorporated into filaments) insecticidal net 331/LN (July 2006) have been published for quality control and the document is available on the WHO Internet homepage at: http://www.who.int/whopes/quality/.

National malaria control programmes and other stakeholders (e.g. the WHO Global Malaria Programme, donors and the manufacturer) have requested WHOPES to assess Olyset as an LN for 5-year household use and for planning replacement cycles. Such consideration should include assessment of bio-efficacy, fabric integrity and longevity. The latter is best assessed through longitudinal studies (requiring a minimum evaluation of 5 years). Noting that such data were unavailable, WHOPES launched a retrospective multi-country cross-sectional survey to assess the bio-efficacy and fabric integrity of surviving nets. The review also included assessment of the

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[1] *Report of the 5th WHOPES Working Group meeting – Review of Olyset Net and bifenthrin 10% WP, 30–31 October 2001*. Geneva, World Health Organization, 2001 (WHO/CDS/WHOPES/2001.4; available at http://www.who.int/whopes/recommendations/wgm/en/).
[2] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at http://www.who.int/whopes/guidelines/en/).

recent publications since 2001, which are referred to in section 2.1.

2.1 Efficacy – background and supporting documents

Several studies on Olyset LN have been published since the full recommendation given in 2001 by WHO. However, only two studies in which Olyset Nets had been in household use for at least 3 years have been published.

Tami et al. (2004) conducted a survey in two villages in the United Republic of Tanzania, 7 years after the distribution of Olyset Nets. The chemical content, bio-efficacy, durability and integrity of the nets were assessed, as well as the desirability. Olyset Nets were distributed between December 1994 and early 1995 and only these nets were followed up in July 2002. In the late 1990s, polyester nets and deltamethrin tablets became largely available in these areas, and the desirability of Olyset Nets was compared with that of ordinary polyester nets, since villagers were familiar with both types of nets. Of the 103 households selected at random, only three no longer possessed Olyset Nets. This reportedly indicated their durability and their value to the population. A detailed household questionnaire was administered to the households still possessing at least one Olyset Net. The owners reported high use rate of Olyset Nets (95%). About 33% of the owners claimed to have re-treated their nets, but this did not correlate with the results of chemical analysis and bioassays. The mean number of holes in 100 nets examined was 13 (range 0 to 70 holes) and the majority of them (55%) were over 2 cm in diameter. Many holes resulted from burns rather than tears. The holes resulting from burns were relatively smaller. The users perceived durability and reduction of mosquitoes and other arthropods as advantages, while the small size of the Olyset Nets (1.1 x 1.8 x 1.5 m) and the big mesh size were considered as disadvantages. However interviewees estimated that Olyset Nets had lost their effectiveness within 6 to 24 month. In comparing their preference for an Olyset Net versus a polyester net (size 1.2 x 1.8 x 1.8m), durability and, to a lesser extent, net integrity and insecticide persistence were considered as advantages. However, the dark green colour, which masks dirt, and the smaller mesh size of the polyester nets were preferred. One Olyset Net per 10 households was

sampled for chemical analysis and bioassays (n = 10). The average permethrin content of the 10 samples was 6.62 g/kg or 33% of the initial target dose (20 g/kg) but with large variation between nets (2.55 to 13.33 g/kg). Nine of the 10 samples induced a knockdown (KD) higher than 95%, but none of the samples induced a mortality $\geq$80% (average mortality 34%). A very weak (non-significant) correlation could be found between the bioassay results and the insecticide concentration (KD60 r^2 = 0.15; mortality r^2 = 0.07). The authors concluded that Olyset Nets after 7 years of use were very much appreciated by the users for their durability, and that their insecticidal activity persisted for at least 7 years. At equal price, half of the interviewees would prefer to buy polyester nets, while the other half would like to buy an Olyset Net.

Two of these Olyset Nets that had been in domestic use for 7 years were retrieved in July 2002 and were stored in plastic bags at room temperature until June 2005, after which they were tested in experimental huts in Muheza (United Republic of Tanzania). The nets caused 59% and 40% mortality against *Anopheles funestus* and *An. gambiae,* respectively (Malima et al., 2008). However no blood feeding inhibition was observed.

In another experimental hut study carried out in Muheza (United Republic of Tanzania) over a six-week period, one Olyset Net with 4 years of domestic use had a similar performance as new Olyset Nets. Mortality was twice as high as that of an untreated net, but blood-feeding rates in the control were low (<20%) and no significant blood-feeding inhibition occurred with either the old or new nets (Maxwell et al., 2006).

A follow-up trial lasting only 2 years was conducted in Kenya (Kisumu area) (Lindblade et al., 2005) and produced interesting results on regeneration after washing. Houses were randomly assigned to one of the six net types, including Olyset net, PermaNet 1.0 and conventionally treated deltamethrin nets. The Olyset Nets had been in storage for 6 years at the time of distribution (Gimnig, personal communication, 2009). These were evaluated for their bio-efficacy by periodic WHO cone bioassays (four times in 2 years). It should be noted that bioassays were not carried out according to the present WHO guidelines: 10 mosquitoes were exposed instead of 5 per cone test; failure was defined at <50% mortality and the knockdown criteria was not considered. Moreover, tunnel tests were not

performed on nets failing the bioassay efficacy criteria. According to the authors' criteria, about 80% (39/49) of Olyset Nets and 77% (44/57) of conventionally treated deltamethrin nets failed in about two years of use. Controlling for cumulative number of washes, the risk of failure of an Olyset Net (Hazard ratio (HR) = 1.29) was not different from a conventionally deltamethrin-treated net, while it was significantly lower for PermaNet 1.0 (HR = 0.14). This apparently poor performance of Olyset Net is largely explained by not considering the knockdown in the bioassays. The average permethrin content of failed Olyset Nets (1118 mg/m²) after two years of use was unexpectedly high and was reported to be the same as the initial target concentration. However, Olyset Nets failing after only 3 months post-distribution (44% mortality in bioassays) showed a mortality of 86% after washing and exposing in plastic bags to the sun for 1–2 days. A laboratory study showed that heating Olyset Nets to 60 °C, but not at 30 or 35 °C, after washing restored the biological activity of these nets (Gimnig et al., 2005). This was in contrast to a previous study (Hougard et al., 2001, in WHO 2001)[1] which indicated full regeneration within two weeks under tropical conditions (temperature 30 °C, RH 80%). It is important to clarify whether regeneration occurs at ambient tropical temperature or if artificial heating is necessary. It is interesting that the Olyset Nets had been washed by their users on average about half as many times as the polyester nets. Of the households studied, 11% reported one or more possible side-effects (rash, runny nose, sneezing, cough) during the first 3 months, but no difference was observed with six other types of net treated either with deltamethrin or permethrin.

In an area in Lao PDR (Khammouane province), the number of malaria cases fell one year after Olyset distribution in 1999-2000 covering 80% of the population (Shirayama et al., 2007). However during the following two years, the number of reported malaria cases returned to the baseline level. Interviews and net inspections were conducted at 240 households after 3 years to understand this increase. About 77% of households still owned

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[1] *Report of the 5th WHOPES Working Group meeting – Review of Olyset Net and bifenthrin 10% WP, 30–31 October 2001*. Geneva, World Health Organization, 2001 (WHO/CDS/WHOPES/2001.4, available at http://www.who.int/whopes/recommendations/wgm/en/).

at least one Olyset Net and 90% used them throughout the year. Some 39% had holes (mean of 2.3 holes per net; range 1–22), or were torn, compared with 36% for ordinary local nets, but no association was found between malaria episodes and absence or presence of holes. There was however a significant association between the manufacturer's recommended washing frequency and malaria episodes.

2.2 Efficacy – WHOPES supervised multi-country study

In a multi-country survey carried out by WHOPES in 2008, in collaboration with the WHO Global Malaria Programme, Olyset Net samples were taken from different sites in seven African countries: Benin, Burkina Faso, Burundi, Cameroon, Côte d'Ivoire, Niger and Togo (Nkuni 2008). The selection of sites in each country was based on the information provided by the manufacturer, as well as information from WHO Regional Offices, and depended on where the distribution, age and use of the nets could be verified. Collection of net samples was not limited to a specific delivery or distribution mechanism. Sites where Olyset Nets were distributed 3 and/or 5 years before the date of sample collection and where nets were likely to have been used year-round were selected for sampling. To ensure a good representation, it was initially foreseen to collect samples in at least three sites per country. However this was not always possible due to logistic reasons.

Sites were initially selected by the Ministry of Health (MOH) and final selection of the sites was done together with the MOH and local partners. Within each selected site, all households were enumerated and randomly selected. For each selected household, the owner's consent to participate in the study and willingness to accept the replacement with a new Olyset Net was obtained. Upon entering a house, the first Olyset Net seen was collected and replaced with a new one. The identity of the net i.e., to be an Olyset Net, was verified by the manufacturer's label and/ or the polyethylene netting material.

Different means were used by the principal investigator to verify the accuracy of the age of the net (e.g., net distribution list of the districts and villages obtained from the National Malaria Control Programme or its partners; special distributions, nets distributed through integrated campaigns; records of birth or

vaccination etc.). Attempts were made to ensure that nets were ± 2 months of 36-month or 60-month age. However, this was not possible in all cases (e.g., in Niger, nets of 60 months were not found and a decision was made to collect those of 48 months of use). In some countries, it was not possible to find nets of the desired ages (± 2 months). "Three-year" nets collected in Burkina Faso and Côte d'Ivoire had actually been in use for 39 and 40 months, respectively. These were grouped and analysed with those nets that were in household use for 3 years.

A questionnaire (data recording form, see Annex IV) to assess the use and washing behaviour of the people was administered in households when the Olyset Net was collected.

Sampled nets were returned to a central site in each country where they were hung or spread on a table for physical inspection, following the requirements specified in the data recording form. Upon completion of the inspection, four sub-samples were taken from each net for bioassays and chemical analyses. A unique five-digit code (the first two for the country and three digits for the net sample) was used to identify each net sample. The samples for bioassay were transferred to the WHO Collaborating Centre in Montpellier[1] (LIN/IRD), France, in cool boxes to limit migration of insecticide to the surface of netting fibres.

The objectives of the multi-country study were to determine the bio-efficacy, the chemical content and the integrity of Olyset Nets after 3 or 5 years of domestic use.

2.2.1 Bioassays

Bioassay was performed on a total of 361 samples of Olyset Nets that were collected from the countries (Table 1). Each net sample was identified with its five-digit survey code.

Nets of 3 or 5 years of use were available in four countries (Benin, Burkina Faso, Burundi, and Côte d'Ivoire). In Niger, nets used for 3 or 4 years were present. In Togo and Cameroon,

[1] Laboratoire de Lutte contre les Insectes nuisibles (LIN), Institut de Recherche pour le Développement (IRD), Montpellier, France.

nets of 3 and 5 years of use were available, respectively. Ten nets were sampled by LIN/IRD in each batch, using a computerized software (Duchon et al., 2009). The numbers of nets subjected to cone bioassays are summarized in Table 1.

In all, 120 nets (about one third of the collected samples) were subjected to WHO cone bioassay. Four samples per net were tested. The net samples were cut from positions 2, 3, 4 and 5 (see Figure 7, Annex IV). The net at position 1 was ignored as it may have been subjected to excessive abrasion and is the part that is supposed to be tucked under the mattress.

Non-blood-fed females (2–5 days old) of *Anopheles gambiae s.s.* (Kisumu strain) were used for bioassays. This is a standard susceptible strain originally colonized in western Kenya. It has been colonized for many years at LIN/IRD and is free of any detectable insecticide-resistance mechanism. The susceptibility of the strain is checked every 3 months using PCR and biochemical assays.

The bioassay results for the four netting pieces from each sampled net were pooled to determine if a net met the WHO efficacy requirement, i.e., ≥80% mortality and/or ≥95% knockdown. One of the four pieces from each net failing the above criteria was selected for the tunnel test. The sample used in the tunnel test was the one which caused mortality closest to the average mortality in the cone bioassay. The WHOPES efficacy criteria for the tunnel test are: ≥80% mortality or ≥90% blood-feeding inhibition.[1] Results of the cone and tunnel tests were considered together to judge on net performance.

Figures 1 and 2 give an overview of the variation of mortality and knockdown, as observed in cone tests, for the different countries and years. Only 33% of the 120 nets tested fulfilled the criteria for the bioassays (40 nets for the KD criteria and only 3 for the mortality criteria). No significant difference was found in the bio-efficacy of Olyset Nets used for either 3, or 4 or 5 years (Chi square p = 0.58). Figure 3 presents the

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[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11, available at http://www.who.int/whopes/guidelines/en/).

relationship between bio-efficacy and total permethrin content in Olyset Netting.

Of the 80 nets failing the bioassay cone test, 49 (61%) fulfilled the WHOPES criteria for the tunnel test (Table 2). However, blood-feeding in some control tunnel tests was low, which reflects a low activity of the batch of mosquitoes used in some tests. Analysis of the data showed that blood-feeding in the controls had a considerable impact on mortality in the presence of treated samples, as the host-seeking behaviour increased the chances of contact with the treated fabric. A cut-off value was obtained at 36% blood-feeding rate in the controls using the Classification and Regression Trees (CART) software. Above this threshold, mortality in mosquitoes with Olyset Nets was significantly higher (77%) than below this (51%). Dirtiness, number of washes, length of use and chemical content of the Olyset Nets had little or no influence on the outcome in terms of mortality using this analytical method. Based on this cut-off value for the controls, only 48/80 (60%) tunnel tests would be valid (Van Bortel et al., personal communication, 2009).

Large differences in the bio-efficacy of Olyset Nets were observed between the countries. When all data of tunnel tests were included in the analysis, the WHO requirements for either the WHO cone test or the tunnel test were met by 71% and 72% of the nets used for 3 and 5 years, respectively (Table 2). A sensitivity analysis was conducted in which the results were adjusted by excluding data from one or more of the tunnel tests that produced less than 36% blood-feeding in the control. As a result, the proportion of Olyset Nets fulfilling WHOPES efficacy criteria increased to 77% for 3 years and 73% for 5 years.

2.2.2 Chemical assays

Chemical assays were conducted (Pigeon 2009a) following the CIPAC method 331/LN/m/3 [permethrin long-lasting insecticidal net].[1] Extraction was done with heptane in a water bath (85–90 °C) for 45 minutes and chromatographic determination by gas chromatography with flame ionization detection (GC-FID).

[1] http://www.cipac.org/.

In all, 361 nets were analysed (Table 1). The mean permethrin content was not different between 3- and 5-year used nets in any of the countries, except for Côte d'Ivoire (Figure 4). The overall mean permethrin content of a 3-year used net was 67% of the initial target concentration of 20 g AI/kg. A 5-year used net (excluding the 4-year used nets of Niger) had a mean permethrin content of 62% of the baseline concentration.

Olyset Nets that complied with the WHOPES LN criteria (cone and tunnel tests combined) had a significantly higher permethrin content than those failing the WHOPES criteria and this was the case for both 3- and 5-year used nets (Table 3).

2.2.3 Washing and physical nature of the nets

At the time of collection, household owners were asked about how many times they had washed their net. During physical inspection, the nets were assigned to one of the four categories of cleanliness i.e., from clean to very dirty (Table 4). Nets were washed infrequently in Burkina Faso and Cameroon, whereas in Benin they were washed more than 20 times during a 3-year period. In almost all countries, over 50% of the nets were scored as dirty to very dirty in year 5 (Table 4). No information was collected on washing products used (detergent, soap).

Differences in the mean number of holes in the nets were observed in different countries after their 3- or 5-year use (Table 5). The mean number of holes per net ranged from 11.6 to 40.7 in 3-year used nets and from 12.0 to 34.7 in 5-year used ones. The nets from Burkina Faso had the fewest holes, whereas the nets in Burundi were heavily torn. In 3-year-old nets, most holes (between 73 and 80%) were small in Côte d'Ivoire, Niger and Togo. In Benin, Burkina Faso and Burundi, between 46 and 57% of the holes of 3-year used nets were small. Most holes were found in the lower part of the nets (Table 5). It is surprising that the number of holes did not apparently increase in 5-year used nets compared with those used for 3 years. The reason for this is not clear but one possibility is that the nets in use did continue to gain more holes with age and were taken out of use and disposed of by the user when they became too torn. This is a weakness of this retrospective design of the study. The mean number of repairs

done per net ranged from zero to 2.6. Only a few holes were caused by burns.

2.3 Conclusions and recommendations

Olyset Net is an LN in which permethrin is incorporated into the filaments. The product is made of knitted high-density polyethylene mono-filament yarn. Olyset received the full recommendation of WHOPES in October 2001,[1] which was before the current WHO guidelines for testing and evaluation of LNs had been published. WHOPES initiated a multi-country survey to assess the bio-efficacy and integrity of Olyset Nets after more than 3 years of use.

Published reports on performance of Olyset Nets used over a period of 3 years or more are scarce. In a cross-sectional survey in the United Republic of Tanzania (Tami et al., 2004), the Olyset Nets retained good bio-efficacy in cone tests even after 7 years of routine household use. The average permethrin content after 7 years was 33% of the initial target dose but with a wide between-net variation of 13 to 67%. Experimental hut trials with these 7-year-old nets induced mortalities between 40% and 59% but no blood-feeding inhibition in mosquitoes. However, in tunnel tests, total blood-feeding inhibition was observed with samples of these nets. Longevity and to a lesser extent fabric integrity were considered by the users as the main advantages of Olyset Nets. Most of the holes were reportedly small and had resulted from burns rather than tears.

In Lao PDR, most of the households still owned an Olyset Net even after 3 years of initial distribution, which showed the good longevity of the net (almost all the owners used the nets throughout the year).

To restore the bio-efficacy of nets after washing, attempted regeneration at 30 °C for 2 weeks was found to be ineffective (Gimnig et al., 2005), but this contrasts with previous findings (WHOPES 2001).[7] Regeneration of bio-efficacy in the field by

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[1] *Report of the 5th WHOPES Working Group meeting – Review of Olyset Net and bifenthrin 10%WP, 30–31 October 2001*. Geneva, World Health Organization, 2001 (WHO/CDS/WHOPES/2001.4, available at http://www.who.int/whopes/recommendations/wgm/en/).

putting the nets in plastic bags exposed to sunlight, as previously recommended by the manufacturer, is impractical. It is still not known how quickly regeneration occurs at ambient tropical temperatures.

In the WHOPES supervised multi-country surveys carried in seven African countries, a large variation was observed between the countries in terms of bio-efficacy and integrity of Olyset Nets.

When survey results were pooled, only 33% of the nets met the criteria based on the cone test alone and no difference was observed between the nets used for 3, 4 and 5 years. The tunnel tests conducted on nets that failed the cone bio-assay raised the proportion of nets meeting the WHOPES criteria to 72%. When adjusting the tunnel test analysis to account for low blood-feeding in the controls, using a cut-off value defined by CART analysis, the proportion of nets fulfilling the WHOPES criteria for bio-efficacy (combined cone and tunnel bio-assays) was 77% and 73% for 3- and 5-year used nets, respectively, which was below the WHOPES threshold of 80%.

The mean permethrin content did not significantly change between 3 and 5 years of use and was about 65% of the initial target concentration (20 g AI/kg) The mean permethrin content in Olyset Nets was significantly higher in nets fulfilling the WHOPES bio-efficacy requirements than in those failing these requirements (Table 3).

As noted above, the integrity of the sampled nets, as measured by inspection of the number and size of holes, was highly variable between the countries (Table 5). Surprisingly, little difference in number of holes per net was observed between the nets used for 3 years and 5 years. The majority of the holes were small (thumb size) and about 75% of them were located on the lower part of the net.

The integrity of LNs in retrospective cross-sectional surveys, such as the one conducted here, may be overestimated since worn-out nets may have been discarded previously and were not likely to be available for evaluation.

Considering the above, the Meeting concluded that:

The bio-efficacy, chemical content and integrity of Olyset Nets were not substantially different between nets that had remained in use for 3 years and those for 5 years. The bio-efficacy of 5-year nets was 73%, which is below the 80% threshold defined by WHOPES;

Noting the extent of published information and the outcome of the WHOPES supervised multi-country survey, the WHO's full recommendation on Olyset Nets for 3 years of use was re-affirmed;

Although the multi-country survey was not designed to measure the longevity of Olyset Nets (i.e., the proportion of nets that remain in domestic use among those distributed at the beginning), the absence of any remarkable increase in the number of holes (integrity) during their 3-year to 5-year use draws attention to the issue of longevity and the possibility that worn-out nets were discarded by the owners during this interval.

Following the review of the available evidence, including the borderline overall bio-efficacy according to WHOPES criteria, the high variability in the average number of holes per net between different countries included in the survey, and the lack of data on longevity of Olyset Nets, the meeting recommended that:

the extension of WHO full recommendation from the existing 3 years to 5 years is not justified;

the national control programmes are strongly encouraged to monitor and evaluate the performance of Olyset Nets and other LNs under local conditions when selecting the most suitable LN for use in their local settings and determining their cycle of replacement.

The meeting also recommended that:

> noting the properties of permethrin as a highly repellent pyrethroid, a better understanding is needed of the relationship between the results of the tunnel test and the experimental hut studies;
>
> further studies on regeneration of Olyset LNs are needed, especially under local operational conditions.

Table 1. **Number of Olyset Nets sampled and the number of chemical assays, cone tests and tunnel tests conducted by country**

Country	Age of nets in years	Nets collected (number of collection sites)	Chemical assays	Cone tests	Tunnel tests
Benin	3	30 (3)	30	10	10
	5	30 (2)	30	10	8
Burkina Faso	3	30 (2)	30	10	1
	5	30 (3)	30	10	5
Burundi	3	30 (9)	30	10	9
	5	30 (8)	30	10	6
Cameroon	3	0	–	–	–
	5	31 (4)	31	10	9
Côte d'Ivoire	3	30 (1)	30	10	10
	5	30 (2)	30	10	8
Niger	3	28 (3)	30	9	6
	4	32 (5)	30	11	7
Togo	3	30 (7)	30	10	9
	5	0	–	–	–
Total	3	178	180	59	45
	4-5	183	181	61	43

Table 2. **Number of Olyset Net samples fulfilling the WHOPES efficacy criteria in cone and tunnel tests and the combined results of cone and tunnel tests. Numbers of nets tested are given in parentheses. Tunnel BF36: validated tunnel tests for a blood-feeding rate of $\geq$36% in the controls.** To avoid any possible bias in the analysis, the samples (denominator) in the combined tunnel/cone test and in the cone test alone were kept the same.

Country	After 3 years				
	Cone	Tunnel	Combined	Tunnel BF36	Combined BF36
Benin	1 (10)	6 (9)	7	2 (2)	–
Burkina Faso	9 (10)	1 (1)	10	1 (1)	10 (10)
Burundi	1 (10)	2 (9)	3	2 (9)	3 (10)
Cameroon	–	–	–	–	–
Côte d'Ivoire	1 (10)	9 (9)	10	9 (9)	10 (10)
Niger	3 (9)	3 (6)	6	3 (6)	7 (9)
Togo	2 (10)	4 (8)	6	None	–
Total	17(59)	25(42)	42 (59)	17 (27)	30 (39)
Acceptance for 3 years			42 (59) 71%		30 (39) 77%

Table 2 (continued). **Number of Olyset Net samples fulfilling the WHO efficacy criteria in cone and tunnel tests and the combined results of cone and tunnel tests. Number of nets tested given in brackets. Tunnel BF36: validated tunnel tests for a blood-feeding rate of ≥36% in the controls.** To avoid any possible bias in the analysis, the samples (denominator) in the combined tunnel/cone test and in the cone test were kept the same.

Country	After 5 years*				
	Cone	**Tunnel**	**Combined**	**Tunnel BF36**	**Combined BF36**
Benin	2 (10)	7 (8)	9	3 (3)	–
Burkina Faso	7 (10)	3 (3)	10	3 (3)	10 (10)
Burundi	4 (10)	3 (6)	7	3 (6)	7 (10)
Cameroon	2 (10)	3 (8)	5	2 (3)	–
Côte d'Ivoire	4 (10)	1 (6)	5	1 (6)	5 (10)
Niger	4 (11)	7 (7)	11(11)	–	–
Togo	–	–	–	–	–
Total	23(61)	24(38)	47 (61)	12 (21)	22 (30)
Acceptance for 5 years			36 (50) 72%		22 (30) 73%

* For Niger, the data refer to samples after 4 years of use.

Table 3. **Mean content of permethrin in Olyset Nets after 3 or 5 years of use according to the acceptance status for bio-efficacy**

Nets tested	**Failed g AI/kg (95% CI)**	**Accepted g AI/kg (95% CI)**	***t* test**
3 years of use: all	11.77 (9.99–13.55)	14.04 (13.09–15.01)	P = 0.0165
3 years of use: excluding nets with BF <36% in tunnel tests	10.92 (8.32–13.52)	13.49 (12.43–14.55)	P = 0.0313
5 years of use: all	10.48 (8.00–13.00)	12.75 (11.89–13.61)	P = 0.0254
5 years of use: excluding nets with BF <36% in tunnel tests	10.11 (7.55-12.67)	12.54 (11.61-13.48)	P = 0.0233

Table 4. **The number of times the Olyset Nets was washed and the general aspect of the nets (Category: 1 = clean; 2 = slightly dirty; 3 = dirty; 4 = very dirty)**

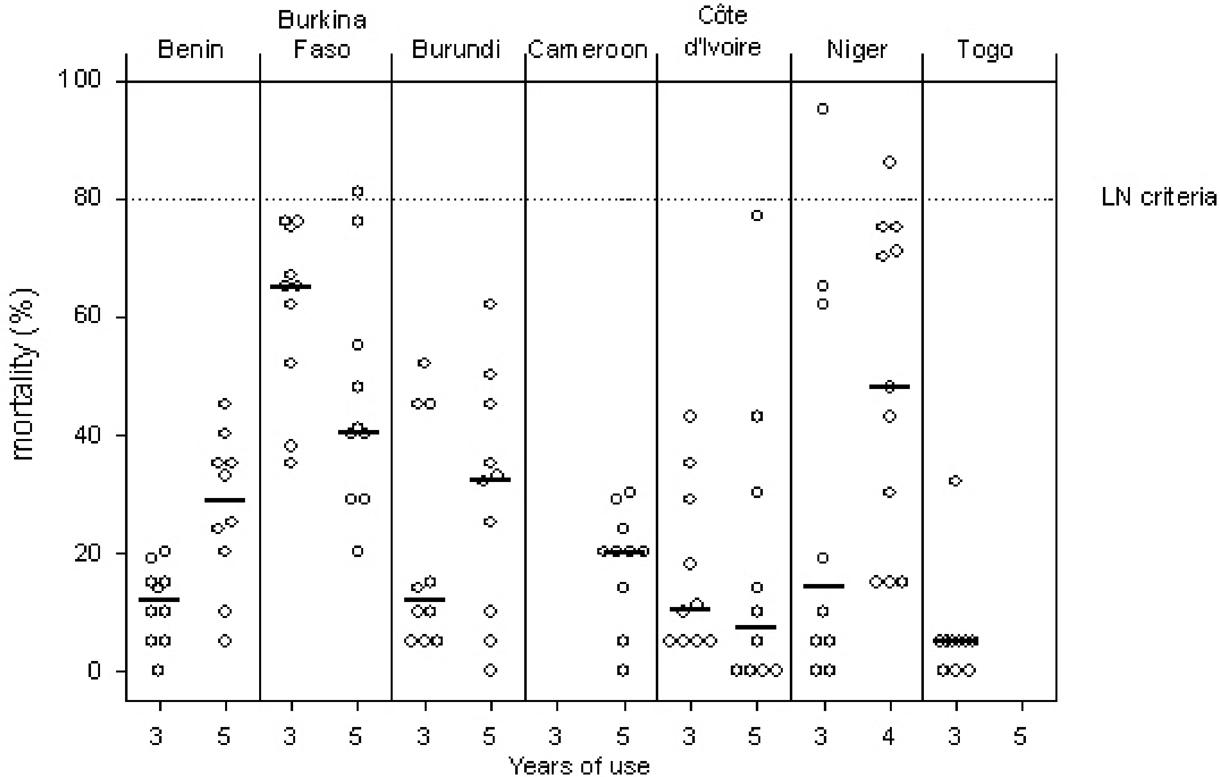
				General aspect of nets (% of nets per category)			
Country	**Year**	**No. of nets**	**Mean no. of washes**	**1**	**2**	**3**	**4**
Benin	3	30	25.8	33	30	27	10
	5	30	50.1	20	17	20	43
Burkina Faso	3	30	6.8	34	23	23	20
	5	30	5.4	27	40	30	3
Burundi	3	30	14.0	34	13	40	13
	5	30	20.4	10	30	53	7
Cameroon	3	0	–	–	–	–	–
	5	31	3.9	10	35	39	16
Côte d'Ivoire	3	30	16.8	10	33	30	27
	5	30	29.5	7	40	46	7
Niger	3	28	18.6	25	46	22	7
	4	32	21.1	9	38	41	12
Togo	3	30	12.2	23	57	20	0
	5	0	–	–	–	–	–

Table 5. **Physical status of Olyset Nets by country and age of net**

Country	Year	No. of nets	Mean no. of holes per net	Percentage of holes of each size[1]			Percentage of holes in each position[2]			Mean no. of open seams
				small	medium	large	lower	upper	roof	
Benin	3	30	12.3	56	28	16	65	28	7	0.3
	5	30	12.0	47	35	18	50	34	16	0.4
Burkina Faso	3	30	11.6	57	38	5	81	12	7	0.1
	5	30	12.9	71	27	3	87	7	6	0.2
Burundi	3	30	40.7	46	43	10	83	9	8	0.3
	5	30	33.8	44	44	12	80	11	9	0.8
Cameroon	3	0	–	–	–	–	–	–	–	–
	5	31	34.7	57	36	7	70	18	12	0.2
Côte d'Ivoire	3	30	14.9	80	19	1	75	16	9	0.1
	5	30	25.2	76	22	2	73	23	4	0.3
Niger	3	28	30.3	73	24	3	83	10	7	0.3
	4	32	28.3	76	22	2	80	11	9	0.1
Togo	3	30	16.6	74	25	2	90	7	3	1.3
	5	0	–	–	–	–	–	–	–	–

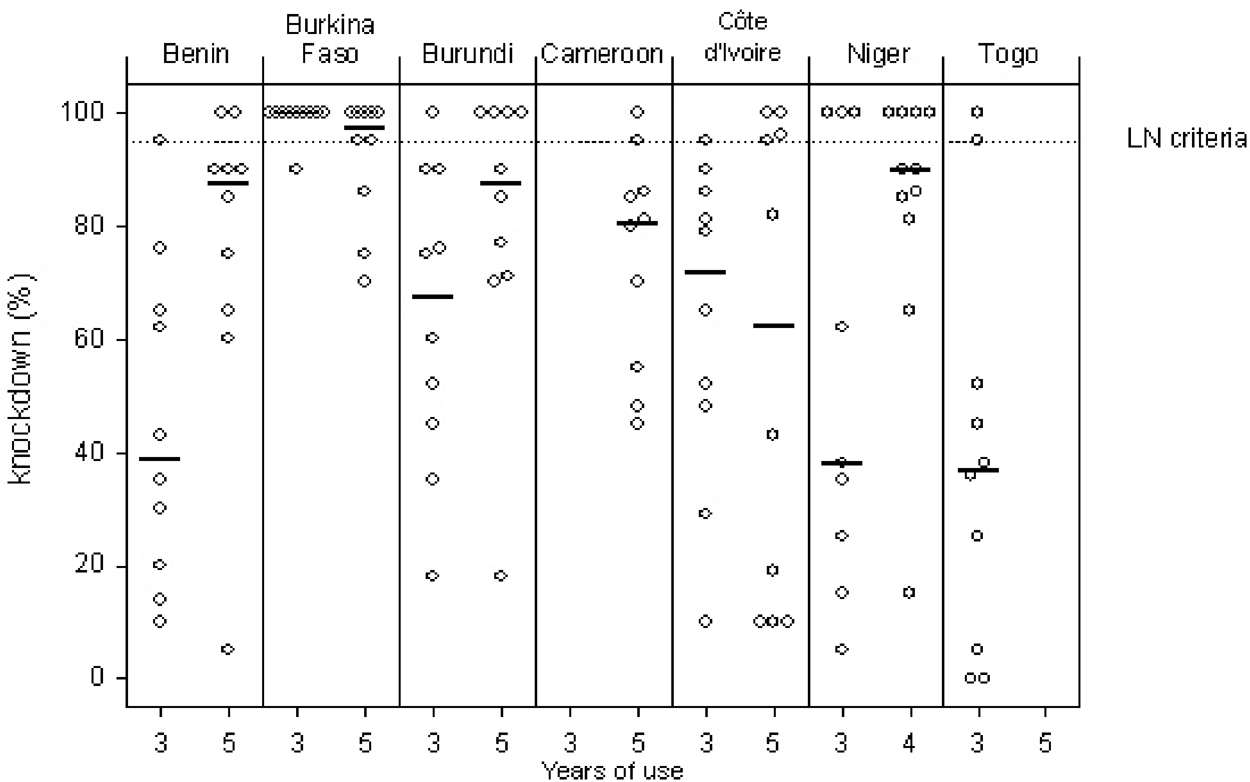
[1]small: hole smaller than will allow a thumb to pass through; medium: a larger hole, but will not allow a closed fist to pass through; large: hole bigger than a closed fist.
[2]lower: lower half of the net; upper: upper half of the net.

Figure 1. **Mortality in mosquitoes in WHO cone tests by country and years of use (3, 4 or 5 years) of nets. The plotted values are the pooled results of mortality for the four netting samples per Olyset Net***



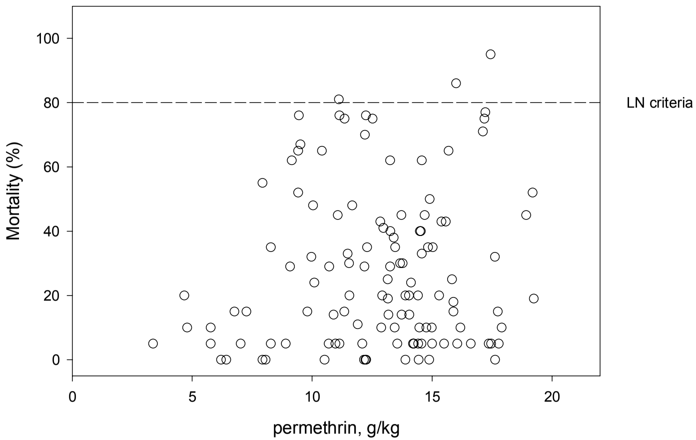
* Each circle represents the pooled results of the mortality for the four netting samples per Olyset Net. Each horizontal line represents the mean mortality for all the nets bio-assayed in each country and for a given year.

Figure 2. **Knockdown in mosquitoes in WHO cone tests by country and years of use (3, 4 or 5 years) of nets. The plotted values are the pooled results of knockdown for the four netting samples per Olyset Net***

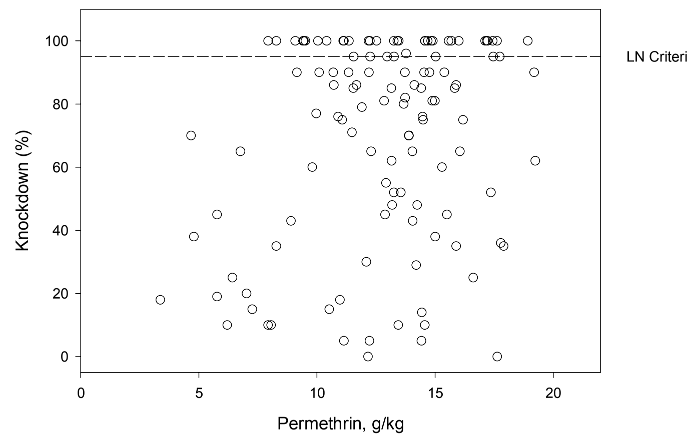


* Each circle represents the pooled results of the mortality for the four netting samples per Olyset Net. Each horizontal line represents the mean mortality for all the nets bio-assayed in each country and for a given year.

Figure 3. **Mortality (A) and knockdown (B) of mosquitoes in WHO cone tests by the permethrin content in nets. Samples for the chemical analysis and the cone bioassays were taken on adjacent pieces of Olyset Netting**

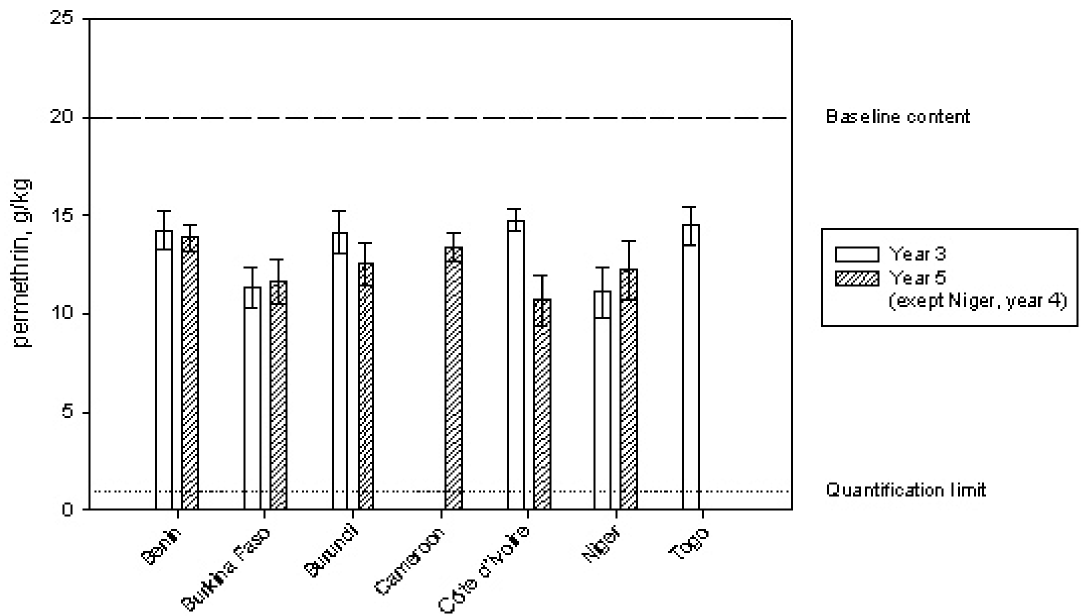


A



B

Figure 4. **Mean permethrin content (with 95% CI) of Olyset Net samples, as measured by GC-FID, according to country and year of net use**



3. REVIEW OF DAWAPLUS® 2.0

The DawaPlus® 2.0 is manufactured by Tana Netting (Thailand) as a deltamethrin long-lasting (coated) insecticidal net. Deltamethrin suspension concentrate (SC) is coated on knitted multi-filament polyester fibres, using a spray-on technology, at the target dose of 2.66 g/kg for 75-denier yarn and 2 g/kg for 100-denier yarn, corresponding to 80 mg deltamethrin per LN m^2, using a polymer as a binder.

Deltamethrin has previously been evaluated by WHOPES for conventional treatment of mosquito nets, at a target dose of 15–25 mg/m^2 AI.[1] The manufacturer has disclosed the nature of the binder used in coating the insecticide and has confirmed that it is the same as the binder used in making another public health insecticide, already subject to the WHO safety assessment (WHO, 2007).[2]

DawaPlus 2.0 is an improved product over DawaPlus®, which was subjected to WHOPES evaluation in 2007 and contained 40 mg deltamethrin per m^2 of netting (WHO 2008).[3]

3.1 Safety assessment

The assessment of the risk to humans of washing and sleeping under DawaPlus 2.0, provided by the manufacturer, was assessed by the Finnish Institute of Occupational Health (FIOH 2008) on behalf of the WHO Programme on Chemical Safety. The WHO document *Generic risk assessment model for*

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[1] *Report of the third WHOPES Working Group Meeting, WHO/HQ, Geneva, 23–24 September 1999*. Geneva, World Health Organization, 1999 (CDS/CPE/WHOPES/99.4, available at http://www.who.int/whopes/recommendations/wgm/en/).

[2] *Safety assessment of K-O TAB 1-2-3 in: Report of the tenth WHOPES Working Group Meeting, WHO/HQ, Geneva, 11–14 December 2006*. Geneva, World Health Organization, 2007 (WHO/CDS/NTD/WHOPES/2007.1, available at http://www.who.int/whopes/recommendations/wgm/en/).

[3] *Report of the eleventh WHOPES Working Group Meeting, WHO/HQ, Geneva, 10–13 December 2007*. Geneva, World Health Organization, 2008 (WHO/HTM/NTD/WHOPES/2008.1, available at http://www.who.int/whopes/recommendations/wgm/en/).

insecticide treatment and subsequent use of mosquito nets (WHO 2004)[1] was used as a guiding document.

The following assumptions/methodologies were used by the proposer in drafting the document:

- no insecticidal treatment of the net by the user is intended, so exposures during treatment or from accidental ingestion of the insecticide were not considered;
- washing was assumed to remove a maximum of 9% of the deltamethrin, rather than the 30% default value used in the generic model;
- the dermal absorption was estimated at 0.2% rather than the 10% default value assumed in the generic model;
- oral exposure from sucking and chewing on the net was considered to be at most 4% of the active ingredient over a 12.5-hour period.

FIOH concluded that the characterization of the risks performed by the proposer closely follows the WHO generic model; where default assumptions are not accepted, justification is presented, mostly in the form of actual experimental data. The conclusion, in line with the generic model, is that no unacceptable exposures were found in maintenance and use of the nets, and that washing or sleeping under them does not pose undue risk to adults, children or neonates.

3.2 Efficacy – background and supporting documents

Supporting data were provided by the manufacturer on the consistency of the net treatment in achieving the target dose, the retention of the insecticide after washing and the efficacy of the net against susceptible mosquitoes after washing (Tana Netting 2009).

[1] *A generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*. Geneva, World Health Organization, 2004 (WHO/CDS/WHOPES/GCDPP/2004.6; available at http://whqlibdoc.who.int/hq/2004/WHO_PCS_04.1.pdf).

To assess the variability of the net treatment procedure for the DawaPlus 2.0, five nets from five different batches were sampled. From each net sample, five sub-samples (30 cm x 30 cm) were taken, one from each side of the net according to the recommended scheme[1] and analysed as a single sample. Deltamethrin concentration was determined by HPLC after extraction. Extraction was done by placing net samples in an Erlenmeyer flask with 100 mL of solvent solution (1:1 mixture of water and solvent solution-1, where solvent solution-1 was a 2:3:5 mixture of acetonitrile:tetrahydrofuran:2-propanol). The flask was placed in an ultrasonic bath for 1 hour and then analysed by HPLC. Separation was done by reversed phase chromatography using isocratic elution. Quantitative determination was made by comparison to an external standard.

It was not possible to conclude on the within-net variability of the deltamethrin content because pieces from the same net were analysed as a pooled sample. Within-batch variability was estimated from the data provided. All nets were within the 2 g/kg ± 25% limits recommended by WHOPES. Between net variability ranged from 3.9% to 8.5% relative standard deviation (RSD).

To estimate the wash resistance and retention index, 25 cm x 25 cm pieces of netting were cut from nine different nets and washed for a specified number of times. The washing method followed the WHOPES recommended protocol. Nets were placed in a bottle with 0.5 L of soap solution (2 g/L Savon de Marseille) and shaken at 155 movements per minute while temperature was maintained at 30 °C. The net pieces were rinsed twice in the clean water under the same conditions, dried at room temperature and stored at 30 °C between the two consecutive washes. Washes were done daily.

WHO cone bioassays were conducted using susceptible, non-blood-fed female *An. gambiae* mosquitoes, Kisumu strain, that were 2–3 days old. After a 3-minute exposure, the mosquitoes were transferred to plastic beakers with access to sugar

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[1] Long-lasting insecticidal nets or netting (LN), Draft Guideline. In: *Manual on development and use of FAO and WHO specifications for pesticides*. (March 2006 revision of the first edition; available at: http://www.who.int/whopes/quality/en/).

solution. Knockdown was measured at 60 minutes and mortality at 24 hours post-exposure. A total of 50 mosquitoes were exposed on pieces from each net. Mortality and knockdown were 100% throughout 20 washes.

The retention index was estimated from chemical analysis of samples from the same nets. The samples were washed 10, 15, 20, 30, 35 or 40 times. Analysis of the total deltamethrin content after washing was determined by HPLC based on WHO Full Specification of Technical Deltamethrin (CIPAC/333/LN). The retention index was estimated by fitting to the model of Hill (1997)[1] and was estimated at 95%.

The total deltamethrin concentration was compared with the bioassay data in order to estimate the minimum effective concentration to meet the WHOPES criteria for knockdown and mortality. These were estimated at 0.48 g/kg and 0.38 g/kg, respectively, and 0.38 g/kg was reported as the minimum effective concentration. Based on the model of Hill (1997), a minimum retention index of 94% was needed to retain the minimum effective concentration to meet the WHOPES criteria after 20 washes. The minimum retention index for DawaPlus 2.0 was therefore set at 94%.

3.3 Efficacy – WHOPES supervised trials

3.3.1 Laboratory studies

Montpellier, France

Laboratory studies were conducted to determine the wash resistance and efficacy of the DawaPlus 2.0 against susceptible *An. gambiae* mosquitoes and to assess the dynamics of insecticide on net fibres (Bonnet et al., 2008). Four DawaPlus 2.0 nets (100 denier) were used in these studies. Ten pieces of netting (25 cm x 25 cm) were cut from each net for the Phase I study (40 pieces total). Eight pieces were used in the regeneration study, 28 were used in the wash resistance and

[1] WHO (2007). *Report of the tenth WHOPES Working Group meeting, 11–14 December 2006*. Geneva, World Health Organization (WHO/CDS/NTD/WHOPES/2007.1, available at http://www.who.int/whopes/recommendations/wgm/en/).

efficacy study, and four pieces were stored at 4 °C as reference samples. All bioassays were done using females of susceptible *An. gambiae* (Kisumu strain, non-blood-fed, 2–5 days old).

The procedures for net washing and bioassays were the same for assessing both regeneration time and wash resistance. Net samples were washed by placing them in 1 L beakers containing a soap solution consisting of 0.5 L of deionized water with 2 g/L of soap (Savon de Marseille, pH 10-11). The beakers were shaken for 10 minutes at 155 movements per minute and at 30 °C. After 10 minutes, the samples were rinsed by placing them in clean deionized water (0.5 L) and shaking them for 10 minutes in the same shaking conditions as above. The rinsing procedure was repeated a second time and then the net samples were dried at room temperature for 2 hours before being stored in aluminum foil in the dark at 30 °C until their next wash.

Bioassays were conducted using the WHO cone test method. Four WHO cones were attached to each piece of netting and 5 *An. gambiae* females were introduced into each cone. Mosquitoes were exposed for 3 minutes and then transferred to separate cages with access to sugar solution. The process was repeated until a total of 50 mosquitoes had been exposed to each net sample. Knockdown was measured 60 minutes post-exposure while mortality was measured 24 hours post-exposure. Results were pooled for analysis so that a total of 200 mosquitoes had been exposed at each time point.

To determine the time required for the regeneration of the DawaPlus 2.0 after washing, WHO cone tests were conducted on nets held for 1, 2, 3, 5 or 7 days at 30 °C after three consecutive washes. Samples from unwashed nets were included for comparison. Mortality and knockdown of mosquitoes were 83% and 97% respectively on unwashed nets. One day after 3 washes, mortality was 78% and knockdown 99%. The differences in mortality and knockdown between the washed and unwashed nets were not statistically significant. No significant regeneration of insecticidal activity was observed in either knockdown or mortality after one week of storage at 30 °C.

Additional tests to study the dynamics of the insecticide on the fibres of the DawaPlus 2.0 were conducted using circular

chamber tests. Non-blood-fed, 2–5 day-old *An. gambiae* (Kisumu strain) mosquitoes were introduced in batches of 13 into a circular chamber 10 cm in diameter and 1 cm in height, forcing contact with the netting material. The time for knock-down in seconds was recorded for each individual mosquito and the MKDT was compared among different net samples. Three replicates of 13 mosquitoes were used for each sample tested. Four netting samples of each net were tested and the results pooled for analysis.

The circular chamber tests were carried out at different intervals (1, 2, 3, 5 and 7 days) on net samples before and after washing 3, 10 or 20 times and held at 30 ^{o}C. MTKD was 704 seconds on unwashed nets. For nets washed 3 times, MTKD was 532 seconds on day 1 and declined to 465 seconds by day 7 after washing. The MKDT on day 7 post-washing was significantly more than that on day 1. For nets washed 10 times, MTKD was 686 seconds on day 1 after washing and declined to 601 seconds on day 7 after washing. For nets washed 20 times, MTKD was 748 seconds on day 1 after washing and declined to 610 seconds on day 7 after washing. The value of MTKD on day 1 was significantly different from that on day 3 after washing of nets that had been washed either 10 or 20 times.

The wash resistance of the DawaPlus 2.0 was measured in WHO cones before and after washing the net samples 1, 5, 10, 15, 20 or 25 times. Mortality of mosquitoes at 0 washes was 76%, declined to 59% after 1 wash and further to 37% after 5 washes. However, mortality after 10, 15, 20 or 25 washes did not fall below 37%. Knockdown remained above the WHOPES threshold (≥95% knockdown) throughout 20 washes, falling to 93% after 25 washes (Table 6). Therefore, it was concluded that the DawaPlus 2.0 met the WHOPES criteria for Phase I testing of LNs and no tunnel test was required.

Deltamethrin contents were measured on all net samples after testing using the CIPAC method 333/LN (Pigeon 2009c). Extraction was done by sonification and shaking in iso-octane/dioxane (4:1) with dipropyl phthalate as an internal standard. Deltamethrin concentrations were determined using HPLC with UV/visible diode array detection. Initial deltamethrin concentrations were 2.03 g/kg for unwashed nets used in the regeneration study and 2.06 g/kg for unwashed nets used in the wash resistance study. These concentrations complied with the

target dose of 2.0 ± 25%. The between-net variation (relative standard deviation) in deltamethrin content was 13.4% at baseline for the regeneration study and 12.7% at baseline for the wash resistance study. Mean deltamethrin concentrations were determined to be 1.42 g/kg after 20 and 25 washes (Table 6 and Figure 5). The overall deltamethrin retention after 20 washes was 69%, corresponding to an average retention per wash of 98%.

3.3.2 Experimental hut studies

3.3.2.1 Malanville, Benin

Experimental hut studies were conducted to assess the efficacy and wash resistance of the DawaPlus 2.0 in comparison to the DawaPlus and a conventionally treated net (Chabi et al., 2009). These studies measure the efficacy of nets in terms of their ability to kill or to inhibit blood-feeding in anopheline vectors of malaria. The studies were conducted in Malanville in north Benin where the wet season occurs from July to December but where irrigation for rice cultivation results in high populations of *An. gambiae* s.l. throughout the year. *An. gambiae s.s.* (M form) is the predominant vector, accounting for 95% of the anophelines found in the area. The remaining 5% are *An. arabiensis*. *An. gambiae* is fully susceptible to permethrin but slightly resistant to deltamethrin (85% mortality in WHO susceptibility tests).

The huts used were of the typical West African style, made from concrete bricks with a corrugated iron roof, a ceiling of polythene sheeting and a concrete base surrounded by a water filled moat to prevent the entry of ants. Mosquitoes were able to enter the huts through four window slits constructed from pieces of metal fixed at an angle to create a funnel with a 1 cm wide gap. The design of the window slits allows for easy entry but greatly limits the egress of mosquitoes once they have entered the hut. A veranda trap made of polythene sheeting and screening mesh (2 m long, 1.5 m wide and 1.5 m high) was fitted at the back of each hut. Mosquitoes were allowed to move unimpeded to and from the veranda trap during the night.

Six different treatment arms were tested as follows:

1. untreated polyester net
2. unwashed polyester net treated with deltamethrin at 25 mg/m^2
3. DawaPlus (previous version with a target dose of 40 mg/m^2) washed 20 times
4. DawaPlus 2.0, unwashed
5. DawaPlus 2.0 washed 20 times
6. polyester net treated with deltamethrin at 25 mg/m^2 and washed until just before exhaustion (3 washes).

All nets were 100 denier polyester nets. The nets were washed in an aluminium bowl containing 10 litres of well water and 2 g/L of soap (like Savon de Marseille). They were manually agitated using a pole and stirred for 3 minutes at 20 rotations per minute, left to soak for 4 minutes and then agitated again for 3 minutes. Nets were rinsed twice in clean water using the same protocol and then dried horizontally in the shade.

For the conventional net washed to just before exhaustion, the exhaustion point was determined by washing a conventional net and conducting a WHO cone bioassay after each wash. Mortality and knockdown fell below WHOPES thresholds (mortality $\geq$80%; knockdown $\geq$95%) after four washes. Therefore, three washes were considered the maximum number of washes before a net was considered exhausted.

Six nets were used for each treatment arm. Before the testing, six holes (4 cm x 4 cm) were made in each net, two on each of the long sides and one on each end. Each week the treatment arms were rotated among the huts and each net from each treatment arm was tested for one night in each hut. Sleepers were rotated through each hut in the night. At the end of each rotation, huts were cleaned and aired to remove potential contamination.

WHO cone bioassays were conducted just before washing, just after washing and after the completion of the hut study. Five cones were affixed to one net from each arm of the study with one cone on each side of the net and one on the top panel. Before washing, mortality and knockdown were 100% for all nets except for the untreated net, where mortality and knockdown were both 0%, and for the DawaPlus 2.0 to be

washed 20 times, where mortality was 96.6% and knockdown was 98.3%.

After washing but before the hut trial, mortality in bioassays on the untreated net was 1.8% and knockdown was 0%. Knockdown was 100% for all other nets except the conventionally treated net washed to exhaustion, where knockdown was 95.1%. Mortality was 100% on all nets except for the DawaPlus washed 20 times, where mortality was 95.7%, and the conventionally treated net washed until just before exhaustion, where mortality was 82.0%.

Similar bioassay results were obtained after the hut trial was completed. Mortality and knockdown on the untreated net were 0%. Knockdown was 100% on all other nets except for the conventionally treated net washed until just before exhaustion, where knockdown was 96.9%. Mortality was 98.4% on the DawaPlus net washed 20 times and 89% on the conventionally treated net washed until just before exhaustion; mortality was 100% for all other nets.

Nets were evaluated over a 12-week period allowing for two full rotations of the Latin square. The sleepers entered the huts at dusk and remained until dawn. Each morning, mosquitoes were collected from inside the net and from the walls and floor of the hut and veranda. Mosquitoes were scored as dead or alive. Live mosquitoes were held for 24 hours to assess delayed mortality. The primary outcomes measured in the experimental hut study were deterrency (reduction in hut entry relative to the control huts), induced exophily (proportion of mosquitoes found in the veranda traps), blood-feeding inhibition (reduction in blood feeding compared with the control huts), and immediate and delayed mortality (proportion of mosquitoes that are killed). Summary data are provided in Table 7.

The total number of mosquitoes entering the control huts was 285. Deterrency in the other huts ranged from 27.3% to -4.2% (an increase) when comparing the total numbers of mosquitoes entering the treated huts relative to the control huts. Highest deterrency was observed in the unwashed conventionally treated nets (deterrency = 27.3%) and the unwashed DawaPlus 2.0 nets (deterrency = 26.6%). However, there were no significant differences among any of the treatment groups.

Exophily in the control huts was 42.8% and was significantly lower than in all other treatment arms. Exophily was highest in the huts with unwashed conventionally treated nets at 64.2%, which was significantly higher than that of unwashed DawaPlus 2.0 (exophily = 53.5%). No other significant differences were observed. The exophily rate in the huts with a DawaPlus 2.0 washed 20 times was 57.6%, which was not statistically different from the conventionally treated net that was washed until just before exhaustion (exophily = 56.9%).

The blood-feeding rate in the control huts was 37.5% and was significantly higher compared with all other treatment groups, where blood-feeding rates ranged from 4.4% to 10.4%. Blood-feeding rates among the treated arms was highest for the conventional nets washed until just before exhaustion (proportion fed = 10.4%) and was significantly greater than blood feeding rates for the DawaPlus (proportion fed = 4.4%) or the DawaPlus 2.0 (proportion fed = 4.8%).

Overall mortality of mosquitoes in the control huts was 4.2% and was significantly lower than the mortality observed in all other huts. In the huts with treated nets, mortality ranged from 61.1% to 74.1%. Mortality was highest in the huts with the unwashed DawaPlus 2.0 (mortality = 74.1%) and was significantly higher than the mortality in the huts with a DawaPlus washed 20 times (mortality = 65.2%), a DawaPlus 2.0 washed 20 times (mortality = 61.1%) or a conventionally treated net washed until just before exhaustion (mortality = 61.2%). The mortality rate in the huts with a DawaPlus 2.0 washed 20 times was not significantly different from that in huts with a conventionally treated net washed until just before exhaustion.

A seventh net for each treatment arm was retained for chemical analysis. Five pieces were cut from each net, total deltamethrin content was determined by HPLC using the CIPAC method 333/LN, and the within-net relative standard deviation (RSD) was calculated (Pigeon 2009b). The analyses were done before washing the nets, after washing was completed and after the hut trial was completed. Before washing, the deltamethrin content on the unwashed DawaPlus 2.0 was 1.84 g/kg (RSD = 25.9%) while that of the DawaPlus 2.0 to be washed 20 times was 1.92 g/kg (RSD = 23.7%). After washing but before the hut trial, the deltamethrin content of the unwashed DawaPlus 2.0

was 2.02 g/kg (intra-net RSD = 16.7%). These concentrations complied with the target dose ± 25%. The deltamethrin content of the DawaPlus 2.0 washed 20 times was 0.83 g/kg (RSD = 26.0%), corresponding to an overall retention of 41%. At the end of the hut trial, the deltamethrin content of the unwashed DawaPlus 2.0 was 2.14 g/kg (RSD = 5.3%). The deltamethrin content of the DawaPlus 2.0 washed 20 times was 0.81 g/kg (RSD = 25.3%) after the completion of the trial (Table 8). For two of the four unwashed DawaPlus 2.0, the within-net RSD was high, indicating a high heterogeneity of deltamethrin on the nets.

3.3.2.2 Muheza, United Republic of Tanzania

The efficacy of the DawaPlus 2.0 was evaluated in veranda trap experimental huts in Muheza (United Republic of Tanzania) against wild, free-flying *Anopheles gambiae* (Tungu et al., 2009). Six treatment arms were included in the study as follows:

1) DawaPlus 2.0, unwashed
2) DawaPlus 2.0, washed 20 times
3) polyester net, conventionally treated with deltamethrin (K-Othrine 10% SC) at 25 mg/m^2.
4) polyester net, conventionally treated with deltamethrin at 25 mg/m^2 and washed to just before exhaustion (three washes).
5) polyester net, conventionally treated with deltamethrin at 25 mg/m^2, washed 20 times
6) untreated net.

All nets used were 100 denier polyester nets. Three were used for each treatment arm. Nets were washed according to a standard protocol. Nets were placed in 10 L of water with 2 g/L of soap (Savon de Marseille) and washed for a total of 10 minutes. During the washing, the nets were agitated for a total of 6 minutes at approximately 20 rotations per minute. The nets were then rinsed twice with water and dried between consecutive washings.

The point of exhaustion was determined by washing three conventionally treated nets (treated at 25 mg/m^2) according to the above protocol and conducting bioassays after each wash and dry cycle. The point of exhaustion was defined as the

maximum number of washes a net could withstand before mortality of mosquitoes exposed in standard WHO cone assays fell below 80% and knockdown fell below 95%. Cone bioassays using *An. gambiae* (Kisumu strain) showed that knockdown on the conventionally treated net fell below 95% after the first wash. Mortality fell below 80% after four washes. The point of exhaustion for the conventionally treated net was therefore set at three washes. The point of exhaustion was also determined for the DawaPlus 2.0. For this net, knockdown fluctuated between 82% and 100% throughout 20 washes, while mortality remained above 90% throughout 20 washes, indicating that the DawaPlus 2.0 met the criteria for Phase I efficacy testing for an LN.

WHO cone bioassays were conducted on all nets that were used in the experimental hut study. The bioassays were done just before the washings, just after the washings, and at the end of the trial. Just before the washings, knockdown and mortality were 100% on all treatment arms except the untreated net, where knockdown and mortality were both 0%.

After washing but before the hut trial, knockdown in bioassays fell to 76% on the conventionally treated net washed 3 times, 64% on the conventionally treated net washed 20 times, 86% on the DawaPlus 2.0 washed 20 times, 92% on the unwashed conventional net, and 90% on the unwashed DawaPlus 2.0. Mortality on these same nets was 84% for the conventionally treated net washed 3 times, 44% for the conventional net washed 20 times, 96% for the DawaPlus 2.0 net washed 20 times, 100% for the unwashed conventionally treated net and 98% for the unwashed DawaPlus 2.0.

At the end of the experimental hut study, knockdown in bioassays fell to 66% on the conventionally treated net washed 3 times, 52% for the conventionally treated net washed 20 times, 82% on the DawaPlus 2.0 washed 20 times, 98% for the unwashed conventionally treated net and 100% for the unwashed DawaPlus 2.0. Mortality on these nets was 66% for the conventional net washed 3 times, 38% for the conventional net washed 20 times, 82% for the DawaPlus 2.0 washed 20 times, 98% for the unwashed conventionally treated net and 100% for the unwashed DawaPlus 2.0.

An additional net in each treatment arm was not tested in the huts but used for chemical analysis by HPLC using the CIPAC method 333/LN (Pigeon 2009d). Five pieces (30 cm x 30 cm) were cut from each net before any washing was done, after washing was completed and at the end of the trial. Before washing was conducted, deltamethrin content ranged from 1.77 to 1.87 g/kg for the DawaPlus 2.0 nets and from 0.43 to 0.47 g/kg for the conventionally treated nets (Table 8). After washing was completed, deltamethrin content on the unwashed DawaPlus 2.0 was 1.82 g/kg while deltamethrin content on the DawaPlus 2.0 washed 20 times fell to 1.09 g/kg, corresponding to an overall retention of 60%. After washings were completed, the deltamethrin content of the unwashed conventionally treated net was 0.53 g/kg. For the conventionally treated nets washed 3 times or 20 times, deltamethrin content after washing was 0.12 g/kg and 0.02 g/kg, respectively, corresponding to an overall retention index of 23% and 4%. At the conclusion of the study, the deltamethrin content of the unwashed DawaPlus 2.0 was 2.01 g/kg, while the deltamethrin content of DawaPlus 2.0 nets washed 20 times was 0.99 g/kg. The deltamethrin content of the conventionally treated nets washed 3 or 20 times was 0.13 g/kg and 0.02 g/kg, respectively while that of the unwashed conventionally treated net was 0.53 g/kg. In all chemical analyses, the deltamethrin content of the unwashed DawaPlus 2.0 fell to within the target specifications of 2.0 g/kg ± 25%.

Before the washings were completed, the relative standard deviation in deltamethrin content of the unwashed DawaPlus 2.0 was 12.4% while the RSD of the DawaPlus 2.0 to be washed 20 times was 8.4%. The RSD of the unwashed conventional net was 13.4% while the RSD of the conventional net to be washed 3 times was 45.3% and the RSD of the conventional net to be washed 20 times was 27.8%. After the washings were completed, the RSD of the unwashed DawaPlus 2.0 was 8.9% and the RSD of the DawaPlus 2.0 washed 20 times was 19.8%. After the washings were completed, the RSD of the deltamethrin content on the unwashed conventionally treated net was 19.3%. The RSD of deltamethrin content on the conventionally treated nets after washing 3 or 20 times was 28.3% and 19.8%, respectively. At the end of the hut trial, the RSD of the unwashed DawaPlus 2.0 was 15.1% while the RSD of the DawaPlus 2.0 washed 20 times was 23.2%. The RSD of the unwashed conventionally treated net at the end of the trial

was 9.7% while the RSD of the deltamethrin content of the conventionally treated nets washed 3 or 20 times was 20.0% and 20.4%, respectively (Table 8).

Six experimental huts were used in the study and were made to the traditional East African veranda trap design. The huts were made of concrete walls smeared with mud and an iron roof with a wooden ceiling lined with hessian cloth. The eaves were open on all sides to allow passage of mosquitoes. Two verandas placed on opposite sides were screened to capture mosquitoes that exited through the eaves or windows while the verandas on the remaining two sides were left open to allow for mosquito entry. The screens on the verandas were rotated periodically to reduce any biases introduced by the position of the veranda screens. The huts were built on concrete plinths and surrounded by a water-filled moat to prevent the entry of ants or other scavengers.

Before the start of the trial, the nets were deliberately holed to simulate a torn net. Six holes, 4 cm x 4 cm were cut in each net, with two holes on each long side of the net and one hole at each end. The three nets per treatment arm and the sleepers were rotated through the huts in a Latin square design. Each net was tested in each hut on at least two nights. Mosquitoes were collected each morning from the floors, walls, exit traps and inside the nets and were scored as dead or alive, blood-fed or unfed. Live mosquitoes were held for 24 hours to assess delayed mortality. For data analysis, the number of mosquitoes captured in the veranda traps was doubled to account for mosquitoes escaping through the open verandas. The trial was run for 54 nights. Summary data are provided in Table 7.

On average, 4.2 *An. gambiae* female mosquitoes were captured each night in the huts with untreated nets. In the huts with treated nets, the number of mosquitoes entering each night ranged from 2.9 to 3.7. However, there were no statistically significant differences in the number of *An. gambiae* captured among any of the treatment arms.

There was a high rate of exophily of *An. gambiae* among all treatment groups. In the huts with untreated nets, 88.9% of all mosquitoes were captured in the exit traps. This was significantly lower than the proportion that were captured in the veranda traps in huts with a conventionally treated net washed

20 times (98.0%), an unwashed DawaPlus 2.0 (96.5%), or a DawaPlus 2.0 washed 20 times (96.3%). Exophily was highest in the huts with a conventionally treated net washed 20 times (98.0%) and was significantly higher than that observed in huts with untreated nets (88.9%), unwashed conventionally treated nets (93.0%), or conventionally treated nets washed until just before exhaustion (94.7%). No other statistically significant differences were observed.

In huts with untreated nets, 21.3% of *An. gambiae* mosquitoes were blood-fed. There were significantly more blood-fed *An. gambiae* in huts with untreated nets compared with huts with unwashed DawaPlus 2.0 nets (10.5%), DawaPlus 2.0 nets washed 20 times (11.0%) or conventionally treated nets washed until just before exhaustion (9.4%). Blood-feeding was highest in huts with conventionally treated nets washed 20 times (24.4%) and was not significantly different from the huts with untreated nets. Blood-feeding was intermediate in huts with unwashed conventionally treated nets (14.5%) and was not significantly different from any other treatment group. Blood-feeding inhibition ranged from 0% to 66.7%. Blood-feeding rates were not significantly different in the huts with the DawaPlus 2.0 washed 20 times compared with the conventionally treated net washed to exhaustion.

Mortality of *An. gambiae* was 0.9% in the huts with untreated nets. This was significantly lower than the mortality observed in all other treatments. Mortality was highest in the huts with unwashed conventionally treated nets (95.6%) and unwashed DawaPlus 2.0 nets (91.2%), and was significantly higher compared with all other treatment groups. The mortality in the huts with the DawaPlus 2.0 washed 20 times (67.7%) was not significantly different from that observed in huts with the conventionally treated nets washed 20 times (57.3%). The overall killing effect for the treated nets ranged from 42.7% to 68.4%.

The DawaPlus 2.0 washed 20 times showed similar efficacy in terms of blood-feeding inhibition and mortality of *An. gambiae* compared with a conventionally treated net washed to the point of exhaustion. It was therefore concluded that the DawaPlus 2.0 met the criteria for Phase II testing.

3.4 Conclusions and recommendations

The DawaPlus® 2.0 is a factory-produced polyester mosquito net treated with deltamethrin SC. The insecticide plus a binder and water is sprayed on nets tumbling in an industrial scale washing machine at the target dose of 80 mg AI/m^2 (2.0 g/kg for 100 denier nets and 2.66 g/kg for 75 denier nets). Deltamethrin SC has previously been evaluated by WHOPES and has been recommended for treatment of mosquito nets.[1]

The manufacturer has disclosed the nature of the binder used in the treatment of the LN and has confirmed that it is the same as the binder used in making the KO-Tab 123 and the DawaPlus that has already been subjected to the WHO safety assessment.

The WHO assessment of the compliance of the manufacturer's assessment of exposure and risks of washing and sleeping under a DawaPlus 2.0 was in line with the WHO generic risk assessment model, although some default values of the guideline were not used but appropriately justified. The assessment concluded that washing of or sleeping under the DawaPlus 2.0 does not pose undue risk to adults, children or neonates.

Laboratory studies demonstrated that, while mortality of mosquitoes in WHO cone bioassays was consistently below the WHOPES criteria of ≥80%, the knockdown rates were ≥95% throughout 20 washes, thus meeting the WHOPES main efficacy criteria of Phase I studies. WHO cone bioassays on DawaPlus 2.0 used in two different experimental hut studies showed a much higher mortality of susceptible *An. gambiae* both before and after washing 20 times, compared with the Phase I study. No obvious explanation could account for the observed differences.

Field studies in Benin and the United Republic of Tanzania demonstrated an equal or greater impact of the DawaPlus 2.0

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[1] *Report of the third WHOPES Working Group Meeting, WHO/HQ, Geneva, 23–24 September 1999*. Geneva, World Health Organization, 1999 (CDS/CPE/WHOPES/99.4, available at: http://www.who.int/whopes/recommendations/wgm/en/).

washed 20 times on mortality and blood-feeding inhibition of prominent malaria vectors, compared with that of the conventionally treated polyester nets (25 mg/m² deltamethrin) washed until just before exhaustion (Table 7). This confirms that the DawaPlus 2.0 fulfils the WHOPES main efficacy criteria of Phase II studies.

Chemical analysis of the deltamethrin content of the DawaPlus 2.0 complied with the target dose of 2.0 g/kg ± 25% as specified by WHO specification guidelines. The Phase I trial showed good homogeneity of deltamethrin content among the nets (RSD ranged from 5.5 to 11.5%). Of six unwashed nets tested in the Phase II trials, two had a high within-net variation of the deltamethrin content (>20% RSD) (Table 8).

Noting the above, the Meeting concluded that:

> the high within-net variability in deltamethrin content in some DawaPlus 2.0 nets will affect the development of standards and methods for quality control of this product; the manufacturer is urged to monitor the variability of deltamethrin content to ensure that it remains in conformity with limits proposed by WHO;
>
> a time-limited interim recommendation be given for the use of DawaPlus 2.0 in the prevention and control of malaria;
>
> WHOPES should coordinate large-scale field studies to confirm the long-lasting efficacy and longevity of the DawaPlus 2.0 and as a requirement for developing full recommendations on the use of the product.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.[1]

[1] WHO specifications for public health pesticides are available on the WHO Internet homepage at http://www.who.int/whopes/quality/en/.

Table 6. **Knockdown and mortality of *An. gambiae* and deltamethrin content and retention (wash curve) of DawaPlus 2.0 in Phase I wash resistance study. Target dose and tolerance limit for deltamethrin in DawaPlus 2.0 LN (100 denier) = 2.0 g/kg ± 25% [1.5 - 2.50 g/kg]**

Wash	KD %	Mortality %	DM content (g/kg)	Between net RSD (%)	DM retention (% of wash 0)	Average DM retention (% at each wash)
0	100	76	2.06	12.7		
1	100	59	2.06	12.1	100.0	100.0
5	100	37	1.90	17.7	92.3	98.4
10	95	42	1.73	11.6	83.8	98.2
15	97	37	1.58	17.2	76.8	98.3
20	98	55	1.42	11.9	69.1	98.2
25	93	41	1.42	8.8	69.1	98.5
					Mean	**98.3**

DM = deltamethrin; KD = knockdown; RSD = relative standard deviation

Figure 5. **Deltamethrin content and retention (wash curve) for DawaPlus 2.0 (WHOPES Phase I)**

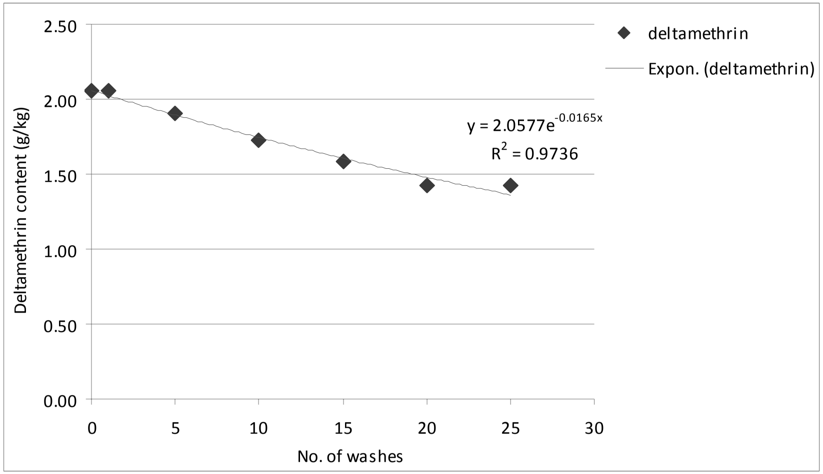

Table 7. **Overview of the experimental hut studies conducted in Benin (*An. gambiae*, M-Form) and the United Republic of Tanzania (*An. gambiae*, S-Form). Values in the same row sharing a same letter superscript do not differ significantly (P >0.05)**

Outcome	Site	Untreated net	DawaPlus 1.0 washed x 20	DawaPlus 2.0 unwashed	CTN unwashed	CTN washed x 20	CTN washed to exhaustion	DawaPlus 2.0 washed × 20
Females/night	Benin	3.9^{a}	3.7^{a}	2.9^{a}	2.8^{a}	–	4.1^{a}	4.0^{a}
	UR Tanzania	4.2^{a}	–	3.2^{a}	2.9^{a}	3.7^{a}	3.2^{a}	3.0^{a}
Exophily (%)	Benin	42.8^{a}	$61.8^{b,c}$	53.5^{b}	64.2^{c}	–	$56.9^{b,c}$	$57.6^{b,c}$
	UR Tanzania	88.9^{a}	–	$96.5^{b,c}$	$93.1^{a,c}$	98.0^{b}	$94.7^{a,c}$	96.3^{c}
Blood fed (%)	Benin	37.5^{a}	4.4^{b}	$6.7^{b,c}$	$5.8^{b,c}$	–	10.4^{c}	4.8^{b}
	UR Tanzania	21.3^{a}	–	10.5^{b}	$14.5^{a,b}$	24.4^{a}	9.4^{b}	11.0^{b}
Mortality (%)	Benin	4.2^{a}	65.2^{b}	74.1^{c}	$69.5^{b,c}$	–	61.2^{b}	61.1^{b}
	UR Tanzania	0.9^{a}	–	91.2^{b}	95.6^{b}	50.2^{d}	$57.3^{c,d}$	67.7^{c}

CTN = conventionally treated net

Table 8. **Deltamethrin content and retention in DawaPlus 2.0 (WHOPES Phase II). Target dose and tolerance limit for deltamethrin in DawaPlus 2.0 LN (100 denier) = 2.0 g/kg ± 25% [1.5–2.50 g/kg]**

a. Before washing

Treatment	Benin		United Republic of Tanzania	
	DM content (g/kg)	Within-net RSD (%)	DM content (g/kg)	Within-net RSD (%)
DawaPlus 2.0, 0 wash	1.84	25.9	1.77	12.4
DawaPlus 2.0, 20 washes	1.92	23.7	1.87	8.4
CTN, 0 wash	0.57	10.1	0.46	13.4
CTN, exhausted	0.56	9.1	0.43	45.3
CTN, 20 washes	–	–	0.47	27.8
DawaPlus, 20 washes	0.96	11.3	–	–
Untreated net	<0.01	–	–	–

CTN = conventionally treated polyester net
DM = deltamethrin
RSD = relative standard deviation

Table 8 (continued). **Deltamethrin content and retention in DawaPlus 2.0 (WHOPES Phase II). Target dose and tolerance limit for deltamethrin in DawaPlus 2.0 LN (100 denier) = 2.0 g/kg ± 25% [1.5–2.50 g/kg]**

b. After washing, before testing

Treatment	Benin			United Republic of Tanzania		
	DM content (g/kg)	**Within-net RSD (%)**	**DM retention (% of wash 0)**	**DM content (g/kg)**	**Within-net RSD (%)**	**DM retention (% of wash 0)**
DawaPlus 2.0, 0 wash	2.02	16.7	–	1.82	8.9	
DawaPlus 2.0, 20 washes	0.83	26.0	41	1.09	11.9	60
CTN, 0 wash	0.58	6.3	–	0.53	19.3	
CTN, exhausted	0.03	22.6	5	0.12	28.3	23
CTN, 20 washes	–	–	–	0.02	19.8	4
DawaPlus, 20 washes	0.53	14.6	56	–	–	–
Untreated net	<0.01	–	–	–	–	–

Table 8 (continued). **Deltamethrin content and retention in DawaPlus 2.0 (WHOPES Phase II). Target dose and tolerance limit for deltamethrin in DawaPlus 2.0 LN (100 denier) = 2.0 g/kg ± 25% [1.5–2.50 g/kg]**

c. After testing

Treatment	Benin		United Republic of Tanzania	
	DM content (g/kg)	**Within-net RSD (%)**	**DM content (g/kg)**	**Within-net RSD (%)**
DawaPlus 2.0, 0 wash	2.14	5.3	2.01	15.1
DawaPlus 2.0, 20 washes	0.81	25.3	0.99	23.2
CTN, 0 wash	0.56	10.2	0.53	9.7
CTN, exhausted	0.04	9.0	0.13	20.0
CTN, 20 washes	–	–	0.02	20.4
DawaPlus, 20 washes	0.45	13.4	–	–
Untreated net	<0.01	–	–	–

4. REVIEW OF TIANJIN YORKOOL® LN

As noted in the Report of the tenth WHOPES Working Group Meeting,[1] apparently similar LN products may be based on different technologies, with the result that a specification developed for one manufacturer's product may not provide a reliable means for testing the acceptability of another manufacturer's product. For this reason, additional information is required to extend existing WHO specifications for LNs to new LN products (i.e., to determine their equivalence) or, where appropriate, to develop separate specifications. The minimum requirements for assessing the equivalence of LNs are currently as follows:

1. The manufacturer must certify to WHO that the active ingredient incorporated into the LN complies with the existing WHO specification for technical material (TC). Where the existing specification has been developed under the new procedure, this means that the active ingredient must be manufactured by a company whose technical material has been evaluated by the FAO/WHO Joint Meetings on Pesticide Specifications (JMPS) and has consequently been recommended for inclusion in the WHO specification for the TC.

2. Laboratory testing to determine regeneration and wash resistance of the candidate LN, as well as its efficacy, is required to be done according to the WHO *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets* (document WHO/CDS/WHOPES/GCDPP/2005.11).[2]

3. The manufacturer must state whether the active ingredient is incorporated within the filament polymer in the spinning process, or is incorporated into a polymer

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[1] WHO (2007). *Report of the tenth WHOPES Working Group meeting, 11–14 December 2006*. Geneva, World Health Organization (WHO/CDS/NTD/WHOPES/2007.1, available at http://www.who.int/whopes/recommendations/wgm/en/).

[2] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (document WHO/CDS/WHOPES/GCDPP/2005.11; available at http://www.who.int/whopes/guidelines/en/).

applied to the outside of filaments; or is applied/incorporated in some other way. If, exceptionally, any detailed information on manufacture of the treated netting is required, it will be treated as confidential by WHO.

4. The manufacturer must provide data to show the applicability of the existing clauses and tests for active ingredient retention/release index in washing and storage stability.

4.1 Objectives

The objectives of this study were to determine the regeneration, wash resistance and efficacy of the candidate long-lasting insecticidal mosquito net of Tianjin Yorkool (China), as part of the requirements for extension of WHO specifications for deltamethrin long-lasting (coated) insecticidal mosquito net.[1] Performance of the Yorkool LN was compared with that of the reference LN for which the WHOPES specification (333/LN, August 2006) was originally developed (namely PermaNet® 2.0) (Rossignol et al., 2009). The evaluation included the following two steps: (i) to determine duration of time required for regeneration of activity of Yorkool LN after washing, in comparison with the regeneration curves of the reference LN; and (ii) to determine wash resistance and bio-efficacy of the candidate LN before and after 1, 3, 5, 10, 20 and 25 washes, in comparison with the reference LN.

4.2 Materials and methods

4.2.1 Net material

The WHO Collaborating Centre in Montpellier, France (LIN/IRD laboratory) received four LNs (75 deniers) from Tianjin Yorkool company. Ten pieces of netting from each Yorkool net were cut and used for the study. Eight of these pieces were used for the regeneration study, 28 for the wash resistance evaluation and the last 4 were kept as reference samples. The corresponding

[1]http://www.who.int/whopes/quality/newspecif/en/http://www.who.int/whopes/quality/deltamethrin_eval_july_2006.pdf).

reference LN (PermaNet® 2.0) was provided by Vestergaard Frandsen Company.

All net samples used during the evaluation were stored at 4 °C after bio-efficacy testing and sent to the WHO Collaborating Centre for quality control of pesticides in Gembloux, Belgium, for chemical residue analysis.

4.2.2 Biological material

Non-blood-fed females of *An. gambiae s.s.* Kisumu strain, a standard susceptible strain originating from Kenya, were used during the evaluation.

4.2.3 Regeneration time and initial efficacy

The time required for full regeneration of biological efficacy was measured using WHO cone tests on six netting samples (four Yorkool + two PermaNet 2.0) washed and dried three times on the same day to deplete surface insecticide and then tested for regeneration at 1, 2, 3, 5 and 7 days after the third wash. Insecticide bio-efficacy curves (24-hour mortality and KD at 60 min), as measured by 3-minute exposure in cone bioassays, were established for the six samples washed three times and compared with six unwashed samples. The number of days to reach an efficacy plateau was considered to be the time required for full regeneration of the net.

Regeneration time studies were supplemented by median knockdown time (MKDT) tests, as described by Skovmand et al. (2008).[1]

4.2.4 Wash resistance

The resistance of the Yorkool LN to washing was determined by cone bioassay tests carried out on netting samples subjected to WHO standardized washing at intervals corresponding to the regeneration time (as determined above). Samples were dried and held at 30 °C between consecutive washes. The bio-

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[1] Skovmand O et al. (2008). Median knock-down time as a new method for evaluating insecticide-treated textiles for mosquito control. *Malaria Journal*, 7:114 (doi:10.1186/1475-2875-7-114).

efficacy evaluation recorded percentage knockdown of mosquitoes at 60-minute interval (KD_{60}), as well as percentage mortality after 24 hours on unwashed samples and after 1, 3, 5, 10, 15, 20 and 25 washes. If the efficacy of a LN sample fell below the cut-off point (i.e., $\geq$80% mortality and/or $\geq$95% KD), a tunnel test was conducted on the netting sample washed 20 times.

4.3 Results

4.3.1 Regeneration time

The regeneration time of Yorkool LN was studied and compared with that of PermaNet 2.0 (Table 9). The mortality was low (55%) for the unwashed Yorkool but it increased to 100% on the first day after the three consecutive wash-dry cycles, and remained at that level on subsequent days of storage. There was an increase in the bio-efficacy shortly after the initial three wash-drying cycles. The time required to reach plateau efficacy, i.e., the regeneration time, was 1 day. KD was maximal (100%) for both the unwashed Yorkool net and on day 1 after washing. Therefore, the regeneration time was considered to be 1 day.

Mortality and KD_{60} with PermaNet 2.0 were 100% both on unwashed nets and on day 1 after washing, as well as on the 7 subsequent days of storage.

In order to better understand the dynamics of the insecticide on the fibres of Yorkool and PermaNet 2.0 after washing, circular chamber tests were carried out at 1, 2, 3, 5 and 7 days of storage (Table 10). The initial average value of MKDT on unwashed Yorkool was 647 seconds. After three washes, the MKDT significantly decreased to 562 seconds on day 1 for the Yorkool net, indicating an increase of efficacy (as previously observed with cone test mortality). During the week of storage, a gradual decrease of MKDT was observed, presumably due to migration of insecticide from the inner of the fibres to the net surface. A value of MKDT significantly different from day 1 was obtained at days 3, 5 and 7. Compared with Yorkool, the initial MKDT of unwashed PermaNet 2.0 was significantly lower at 462 seconds. During storage, a gradual decrease was

observed on samples washed three times (i.e., from 396 seconds at day 1 to 286 seconds at day 7).

4.3.2 Wash resistance and efficacy

The results of bioassays carried out on unwashed and washed LNs are presented in Table 11. The % KD of Yorkool net was always higher than the WHO threshold (i.e. ≥ 95% KD) until 20 washes. At 25 washes, the KD effect decreased to 96% but was still higher than the WHO threshold. The mortality of mosquitoes with the unwashed Yorkool net was low (55%). But from the first wash until 20 washes, the mortality with Yorkool was always higher than the WHO threshold, i.e., close to 100% during the first 15 washes and 93% at 20 washes. Therefore the Yorkool net required one wash to reach full efficacy and retained it until 20 washes. After 25 washes, mortality fell to 48%. The manufacturer is invited to explain the cause of the increase in mortality after the first wash.

For PermaNet 2.0, the KD was very high, i.e., 99-100%, up to 25 washes. The mortality with PermaNet 2.0 was 100% for the unwashed samples and samples washed up to 10 times, then decreased slightly but was still higher than the WHO threshold even after 25 washes.

4.3.3 Chemical assays

Summary data are presented in Table 12 and Figure 6. The deltamethrin content (2.11 g/kg) in the unwashed Yorkool net complied with the target dose of 1.8 g/kg (± 25%) (Pigeon 2009e). The between-net variation, expressed as the relative standard deviation (RSD) of the content found on four single samples taken from the four nets, was 3.9% and showed good homogeneity of the AI content of different nets. The deltamethrin R-isomer content in all Yorkool net samples was lower than the limit of quantification (LOQ) (<0.01 g/kg). The average deltamethrin content was 0.86 g/kg after 10 washes and 0.46 g/kg after 20 washes. The overall deltamethrin retention after 20 washes was 22% corresponding to an average retention per wash of 92%, which was comparable to that of the retention index of PermaNet 2.0 (93%).

4.4 Conclusions and recommendations

The Yorkool net showed high efficacy against susceptible *Anopheles gambiae* in Phase I laboratory bioassays. KD of mosquitoes remained above 95% and mortality above 80% for 20 washes, and therefore Yorkool met the WHO criteria for the Phase I study.

The Yorkool LN needed one wash to be fully effective, while this was not the case for the reference LN.

The deltamethrin content of the Yorkool LN (1.8 g/kg ± 25%) and the average retention per wash (92%) were similar to the values of the reference LN (93%). The between-net variation of the deltamethrin content was within the limits specified in WHO guidelines.[1]

The study of insecticide dynamics using MKDT chamber tests provided supplementary information on the regeneration of activity after washing. Because the bio-efficacy using WHO cone assays was 100%, it was not possible to detect any significant variation between day 1 and 7 of storage after washing. However, during that time, the average MKDT gradually decreased indicating an incremental improvement in bio-efficacy during this interval. This trend in MKDT was similar between Yorkool LN and the reference product.

Considering the above, the Meeting concluded that:

> the bio-efficacy of Tianjin Yorkool LN is comparable to the reference product for which WHO specifications for deltamethrin long-lasting (coated) insecticidal net have been developed.

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[1] FAO/WHO (2006). *Manual on development and use of FAO and WHO specifications for pesticides*. Revision of the 1st Edition. Available only on the Internet at http://www.who.int/whopes/quality/en/.

The Meeting recommended:

> extension of WHO specifications for deltamethrin long-lasting (coated) insecticidal net to Yorkool LN, subject to satisfactory assessment of the physical and chemical properties of the product by the FAO/WHO Joint Meeting on Pesticide Specifications (JMPS).

Table 9. **Regeneration time as determined by average percentage mortality (%M) and knockdown (%KD) of *Anopheles gambiae* females in bioassays of unwashed and 3-times washed nets stored at 30 °C for 1 to 7 days, for the Yorkool LN in comparison with the reference LN**

LN	**Un-washed**		**3 washes + 1 day**		**3 washes + 2 days**		**3 washes + 3 days**		**3 washes + 5 days**		**3 washes + 7 days**	
	% M	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**
Yorkool	55	100	100	100	100	100	100	100	94	100	100	100
PermaNet 2.0	100	100	100	100	100	100	100	100	100	100	100	100

Table 10. **Median knockdown times (average and confidence interval in seconds) of *Anopheles gambiae* females exposed to unwashed and 3-times washed nets stored at 30 °C for 1 to 7 days, for the Yorkool LN in comparison with the reference LN**

LN	Un-washed	3 washes + 1 day	3 washes + 2 days	3 washes + 3 days	3 washes + 5 days	3 washes + 7 days
Yorkool	647	562	556	438	365	436
	(601–693)	(542–582)	(535–577)	(419–457)	(348–382)	(419–455)
PermaNet 2.0	462	396	346	351	295	286
	(444–480)	(374–418)	(328–374)	(326–376)	(281–309)	(278–294)

Table 11. **Wash resistance as determined by average percentage mortality (%M) and knockdown (%KD) of *Anopheles gambiae* females in bioassays of unwashed and 1- to 25-times washed nets, for the Yorkool LN in comparison with the reference LN**

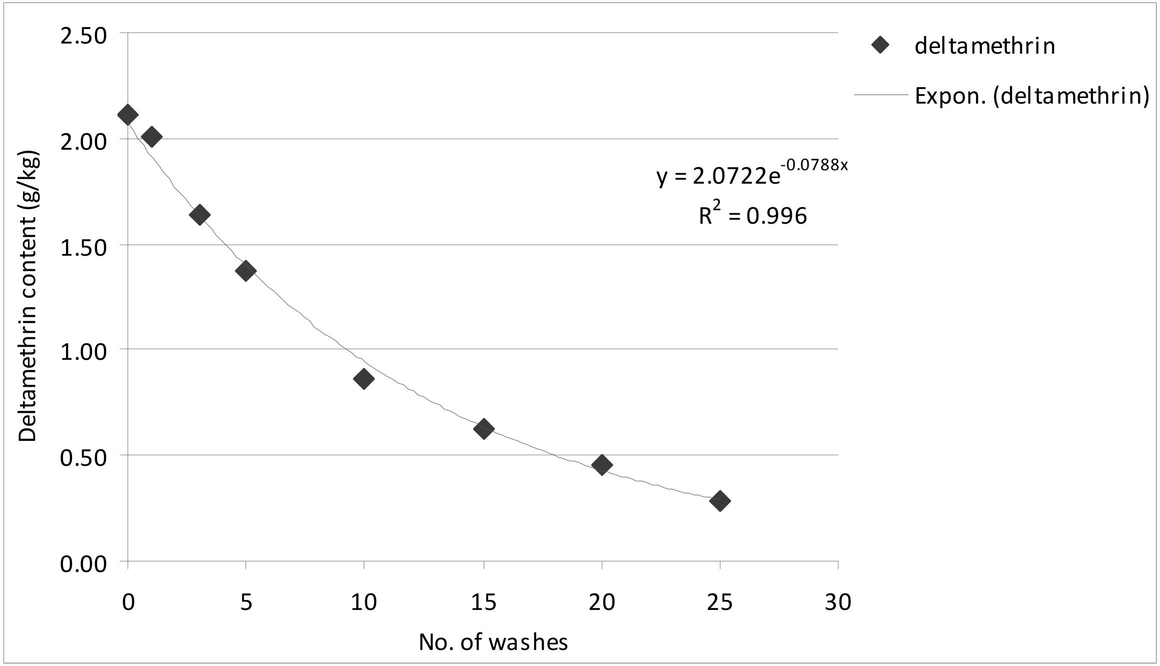
LN	**Un-washed**		**1 wash**		**3 washes**		**5 washes**		**10 washes**		**15 washes**		**20 washes**		**25 washes**	
	% M	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**	**% M**	**% KD**
Yorkool	55	100	99	100	99	100	99	100	100	100	98	100	93	99	48	96
PermaNet 2.0	100	100	100	100	100	100	100	100	100	100	96	100	92	99	83	100

Table 12. **Deltamethrin (DM) content and retention (wash curve) of Yorkool LN in a laboratory wash resistance study (WHOPES Phase I). (Target dose and tolerance limit for deltamethrin in Yorkool LN (75 denier) = 1.8 g/kg ± 25 % [1.35–2.25 g/kg])**

Wash	DM content (g/kg)	Between net RSD (%)	DM retention (% of wash 0)	Average DM retention (% at each wash)
0	2.11	3.9		
1	2.00	2.2	94.8	94.8
3	1.64	3.2	77.4	91.8
5	1.37	5.7	64.8	91.7
10	0.86	7.3	40.8	91.4
15	0.63	18.7	29.7	92.2
20	0.46	7.1	21.6	92.6
25	0.29	21.9	13.6	92.3
			Mean	**92.0**

RSD = relative standard deviation

Figure 6. **Deltamethrin content and retention (wash curve) for Yorkool LN (WHOPES Phase I)**



5. GENERAL RECOMMENDATIONS

Since the time of WHOPES recommendation for the first LN in 2001, millions of insecticide-treated nets (ITNs) and LNs have been distributed by national malaria control programmes and other agencies in a large number of endemic countries for the prevention and control of malaria. Several LN products have received WHOPES interim or full recommendations and many new products are in the research and development pipeline. LNs recommended by WHOPES so far use polyester or polyethylene fibres, various insecticides and concentrations, and various manufacturing technologies. However, little is known of the longevity, physical integrity, attrition rate, and persistence of bio-efficacy and chemical content of different types of LNs under the operational conditions in different settings that may have varying human behaviour and cultural practices of net usage. In the absence of such useful information, it is impossible for the donor, procurement and user organizations/agencies to make appropriate decisions, particularly on planning replacement cycles. The thirteenth Meeting of the WHOPES Working Group dwelt on these issues in considerable detail and made the following recommendations:

1. Much more widespread longitudinal monitoring and evaluation of LNs should be introduced as a routine measure in all medium- and large-scale ITN and LN programmes. Such monitoring and evaluation should be regarded as a necessary process of good implementation practice. The resulting data will provide information for country-specific decisions in procurement and replacement cycles and in the interpretation of data on the local impact of vector control. It will also assist WHOPES in developing or refining recommendations. This monitoring and evaluation should use guidelines and methods to be developed jointly by the WHO Global Malaria Programme (GMP) and WHOPES. In the implementation of the activity, GMP and WHOPES should promote networking and collaboration between national programmes and local research institutions, and it should *inter alia* cover the following issues:

   - Longevity (attrition rate): what proportion of nets introduced into domestic use are still in use or available

for use (not yet lost, abandoned or destroyed) for up to 5 or more years from the beginning of their use?

- Integrity: at what rate do holes accumulate in the netting fabric, and what environmental/human behavioural factors influence this?

- Bio-efficacy: what is the bio-efficacy of the LN after certain periods of use?

- Retention of chemical content: compared with the initial target dose, what percentage of the insecticide AI is retained in the LN fabric with a number of years of use and what are the between-net variations in AI content?

- Insecticide resistance: what is the impact of the use of LN on the susceptibility of the local target vector species?

- Efficacy assessment: investigate how the gradual accumulation of holes and the gradual loss of insecticide interact to make the net less effective in (a) conferring personal protection, and (b) reducing the survival of malaria vector populations.

2. In the FAO/WHO Manual on development and use of FAO and WHO specifications for pesticides (draft guideline specifications for LNs),[1] it is proposed that for the purpose of chemical analysis, the analytical method and the number and size of test portions analysed should be designed to provide results with a relative standard deviation (RSD) of ≤ 5%. In this application, the 5% is a measure of required precision for the estimate of the average content of insecticide in the net. By contrast, the WHOPES Phase II trials are designed to evaluate, among other parameters, the within-net variability of the active ingredient content, expressed as the RSD of the active ingredient content measured on five individual pieces of a net taken according

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[1] FAO/WHO (2006). *Manual on development and use of FAO and WHO specifications for pesticides*. Revision of the 1st Edition. Available only on the Internet at http://www.who.int/whopes/quality/en/.

to the WHO recommendations for LN sampling. This variability is very often incorrectly assessed in terms of the 5% criterion, which is not applicable for this measure of within-net variation.

Instead, and on the basis of the chemical analysis and efficacy results obtained during the last WHOPES Phase II trials, the Meeting proposed a criterion of 20% as the maximum acceptable RSD for the active ingredient content measured in five individual net pieces of 25 cm x 25 cm taken according to the above-mentioned FAO/WHO Manual. The Meeting recommended that this proposition be considered and discussed by the FAO/WHO Joint Meeting on Pesticides Specifications (JMPS).

ANNEX I. WHOPES RECOMMENDATIONS ON PERMANET® 3.0

On 23 January 2009, Vestergaard Frandsen (VF), the manufacturer of PermaNet® 3.0, informed WHOPES of its amendment to the product claim from the former two-in-one product “intended for counteracting resistance” to one of “improved bio-efficacy against resistant mosquitoes” (the “first revision”). In a subsequent open letter, dated 8 April 2009, to the participants of the 12th WHOPES Working Group (the “12th WG”) Meeting, held in WHO/HQ on 8-11 December 2008, the manufacturer further revised the claim to one of “improved efficacy against pyrethroid-resistant malaria vectors” (the “second revision”) and requested a re-evaluation of PermaNet 3.0 in the light of the new claims.

The 12th WG Meeting had made the following recommendations and conclusions:

> that a time-limited interim recommendation be given for the use of PermaNet 3.0 in the control and prevention of malaria;
>
> that WHOPES coordinates large-scale studies (WHOPES Phase III studies) of PermaNet 3.0 to confirm its long-lasting efficacy, fabric integrity and community acceptability as a requirement for developing full recommendations on the use of the product.

Following a review of the available evidence, the 12th WG Meeting concluded:

> that PermaNet 3.0 cannot be considered as a tool to control mosquito populations resistant to pyrethroids or to prevent the spread of pyrethroid resistance. However, the Meeting commended the manufacturer for its initiative in developing tools to control pyrethroid-resistant mosquitoes and encouraged it to conduct further research and development in this area.

The 13th WHOPES Working Group (the “13th WG”) Meeting noted that there is no provision for appeal or challenge against

WHOPES recommendations. However, comments made by the applicant or other parties may be discussed and considered at a subsequent WHOPES Working Group meeting.

The 13th WG was, therefore, requested to:

1. consider the new claims;
2. advise WHO on the possible/potential use of PermaNet 3.0 in malaria prevention and control in the light of the new claims.

By way of background, the Meeting was reminded that:

> Industry applicants submitting a formal application for the evaluation and testing of pesticide products are - at the outset of the process - fully informed of the relevant WHOPES guidelines, projected timeframe and likely cost of the evaluation and testing. In the case of PermaNet 3.0, VF was informed of, and had agreed to, the requirements, procedures and criteria for WHOPES testing and evaluation of PermaNet 3.0 from the outset, including the study protocols.
>
> WHOPES made its recommendations on PermaNet 3.0 using its scientific judgment and on the basis of all available evidence. In accordance with the established WHOPES procedure, VF was, during the evaluation process, given an opportunity to provide technical input into the study reports relating to the above-mentioned product. This technical input was considered by the 12th WG Meeting.

The 13th WG reviewed PermaNet 3.0 in the light of the first revision to the claim. PermaNet 3.0 utilizes deltamethrin and a synergist, piperonyl butoxide (PBO). PBO synergizes oxygenase- and esterase-based resistance mechanisms, which are important mechanisms in *Culex quinquefasciatus,* the nuisance mosquito and filariasis vector. Prior to the commencement of the WHOPES field trials, evaluation of PermaNet 3.0 against *Culex* was welcomed by the manufacturer and the first revision retained consideration of *Culex*. The second revision effectively removed any consideration of *Culex*. Contrary to the manufacturer's opinion, the WG considers the studies reported on *Culex* to be highly relevant. For PermaNet 3.0 to be a significant advance on

earlier technology, it should show higher efficacy against pyrethroid-resistant *Culex* than PermaNet 2.0, which lacks PBO.[1] Three trials were considered by the 12th WG against *Culex* (one of which was sponsored privately by the manufacturer) and each one showed little or no improvement in efficacy of PermaNet 3.0 over PermaNet 2.0. Thus there is no evidence of improved efficacy of PermaNet 3.0 against *Culex* mosquitoes with multiple pyrethroid-resistance mechanisms.

The 13th WG went on to consider the second revision to the claim. It concluded that the claim of improved bio-efficacy against pyrethroid-resistant malaria vectors is too broad and the evidence does not support the claim. The claim does not specify any resistance mechanism and comparison product against which the efficacy might be judged. Without a more specific claim and target product profile, it is not possible to produce a suitable protocol, test procedure and criteria. The Meeting also noted that, in order to demonstrate the new product claim of improved efficacy, the test guidelines would require a more rigorous control, such as the product minus the synergist. Without such controls the evidence cannot support the new claim of improved efficacy. Comparison in experimental huts may be a suitably controlled method to measure any improved efficacy (mortality and blood-feeding inhibition) against target species with known resistance mechanism in different settings. Comparison of the company's existing WHOPES-recommended LN and 20-times washed LNs should also be included to ensure that efficacy and product claim are not overestimated.

Taking into account all the available evidence, the 13th WG Meeting confirmed the earlier conclusion and recommendations made by the 12th WG. In addition, the 13th WG concluded that the second revised claim of improved bio-efficacy against pyrethroid resistant malaria vectors is too broad and that the available evidence does not support this claim.

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[1] It is incorrect to assume that *Culex* is inherently capable of tolerating exposure to ITNs. ITNs treated with non-pyrethroids (e.g. organophosphates, carbamates, pyrroles) have induced high mortalities against *Culex* in experimental hut trials.

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ANNEX IV. QUESTIONNAIRE – WHOPES LARGE-SCALE TESTING AND EVALUATION OF OLYSET

Five digit survey code (first two digits country; one digit village; two digits for sample: -------------------

Country ………………………
Province ……………………
District …………………… …
Village ………………………..
Nearest town ……………………

Date of survey (DD/MM/YY) ………………………………….

Specify exact location of household in the village ………………
…………………………………………………………………….

Batch number of the net sampled (if readable)……………….
(attach the LN label to the form)

Date of production of the net (if readable) …………………………….

Information on net usage provided by:

1) User of this net
2) Caretaker of those using the net
3) Head of household
4) Other (specify) ……………………………

Date of receipt/purchase of the net: ------------- (month)

Information on net usage:

1) Year-round and every night.
2) Year-round but occasionally.
3) Seasonally but every night.
4) Seasonally and occasionally

How is the net used?

1) Hanging over the bed
2) Hanging over sleeping mat/mattress on the ground
3) Other (specify) ……………………………

How did you get the net?

1) Paid or purchased yourself?
2) Given free
3) Specify

When was the last time you washed the net? …………(month)

How frequently do you wash the net? …………………(month)

How many times a net is washed in a year? ……… times

How was the net last washed?

Water:

1) cold …. 2) warm …. 3) hot ….

Soap:

1) village (local)-made soap
2) commercial bar
3) commercial powder
4) mix of soap and powder

Soaking:

1) yes …. 2) no ….
If yes, how long? ……. (hour)

Rinsing:

1) yes …. 2) no ….

Rubbing against rocks/stone:

1) yes …. 2) no ….

Where was the net dried?

1) inside … 2) outside …

If dried outside:

1) in shade ….2) in sun ….

Physical inspection of the net

Does the net have holes?

1) yes … 2) no …

If yes, use the following code for sizes of holes:

1) hole smaller than will allow a thumb to pass through
2) a larger hole, but will not allow a closed fist to pass through
3) hole bigger than a closed fist

Total number of holes:

…….. total size 1
…….. total size 2
…….. total size 3

Total number of holes:

…….. lower half of the net
…….. upper half of the net
…….. roof

Total number of open/failed seams using the size coding provided above:

…….. total size 1
…….. total size 2
…….. total size 3

Total number of repairs:

….. with stitches
….. with knots
….. with patches

Total number of holes due to burns: ………

Aspect of net:

1) clean
2) slightly dirty
3) dirty
4) very dirty

Name and signature of investigator:

…………………………………………………

Figure 7. **Sample preparation for determination of permethrin content and biological assays (position 1 was ignored as it may have been subjected to excessive abrasion and is the part that is supposed to be tucked under the mattress)**