Comparison of the efficacy of long-lasting insecticidal nets PermaNet® 2.0 and Olyset® against *Anopheles albimanus* under laboratory conditions

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Insecticide-treated nets provide a reduction in human-vector contact through physical barrier, mortality and/or repellent effects that protect both users and non-users, thereby protecting the wider community from vector-borne diseases like malaria. Long-lasting insecticide-treated nets (LLINs) are the best alternative. This study evaluated the bioefficacy of LLINs PermaNet® 2.0 and Olyset® under laboratory conditions with Anopheles albimanus. *The laboratory strain was evaluated for insecticide susceptibility with selected insecticides used for malarial control. Regeneration time and wash resistance were evaluated with the standard bioassay cone technique following WHO guidelines. Heat assistance was used for Olyset® nets; the nets were exposed to four different temperatures to speed the regeneration process. The regeneration study of PermaNet® 2.0 showed that efficacy was fully recovered by 24 h after one and three washes and wash resistance persisted for 15 washes. Regeneration of Olyset® nets was not observed for nets washed three times, even with the different temperature exposures for up to seven days. Thus, for Olyset® the wash resistance evaluation could not proceed. Differences in response between the two LLINs may be associated with differences in manufacturing procedures and species response to the evaluated LLINs. PermaNet® 2.0 showed higher and continuous efficacy against* An. albimanus.

Key words: insecticide-treated bednets - mosquito control - biological assay

Malaria is considered to be the most prevalent vector-borne disease worldwide. For its prevention, it is necessary to have an appropriate and timely care program for patients and successful integrated and selective control of the vector species (Rafinejad et al. 2008). This control in highly endemic countries relies largely on one of two main methods: insecticide-treated (mosquito) nets (ITNs) and indoor residual (house) spraying. Both methods are known to be highly effective and current evidence suggests that they are very similar in their relative impact on malaria incidence (Yukich et al. 2008).

Given the increasing availability of resources for malaria control, the Roll Back Malaria Partnership set an ambitious target for 2010 of 80% protection of high-risk groups by a "locally appropriate" vector control measure (RBM 2005). As a consequence of this decision, ITNs are being implemented as part of national malaria control programs around the world (Lindblade et al. 2005). ITNs lead to a reduction of human-vector contact and provide a physical barrier; the scale-up will not only protect users, but also non-users through insecticidal and/or repellent effects gained with high coverage levels that benefit the whole community (Rafinejad et al. 2008, Gu & Novak 2009).

There are two categories of ITNs: conventionally treated nets and long-lasting insecticidal nets (LLINs). A conventionally treated net is a mosquito net that has been treated by dipping in a World Health Organization (WHO) recommended insecticide. To ensure its continued insecticidal effect, the net should be re-treated regularly, usually after about three washes or at least once a year. A LLIN is a factory-treated mosquito net made with netting material that has insecticide incorporated within or bound around the fibres. The net must retain its effective biological activity without re-treatment for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions (WHO 2005).

Pyrethroids are the only class of insecticides that are currently recommended for use on mosquito nets. Treated nets should be non-toxic for children and non-irritant when in contact with exposed skin. The insecticides recommended by the WHO Pesticide Evaluation Scheme (WHOPES) are alpha-cypermethrin (10%), cyfluthrin (5%), deltamethrin (1-25%), etofenprox (10%), lambda-cyhalothrin (2.5%) and permethrin (10%) (Zaim et al. 2000, Nagera & Zaim 2004).

Three LLINs have been fully recommended by the WHO and are now commercially available: Olyset®, PermaNet® 2.0 and Yorkool LLIN (WHO 2010). An additional six LLINs have an interim recommendation from the WHOPES for the prevention of malaria: DawaPlus® 2.0, PermaNet® 2.5, PermaNet® 3.0, Netprotect®, Duranet® and Interceptor®. Data on the efficacy of the interim recommended nets remains limited (Banek et al. 2010, WHO 2010).

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With the development of new LLINs and long-lasting treatments by industry, it is important to apply standardized testing procedures and to accelerate evaluation, in the laboratory and the field, of new and already established products to ensure the shortest time between development and use by at-risk populations (Kulkarni 2006).

In this study, we evaluated the bioefficacy of PermaNet® 2.0 (Vestergaard Frandsen) and Olyset® (Sumitomo Chemicals) under laboratory conditions with one of the most important Latin American malaria vectors, *Anopheles albimanus*. Regeneration time, which gives an indication of the duration required following washing for the surface chemical content to replenish, was evaluated for both nets.

MATERIALS AND METHODS

Nets evaluated - Olyset® is a polyethylene net in which permethrin is incorporated (1.000 mg/m²); it was approved by the WHO in 2001. The second LLIN to receive a full WHO recommendation was PermaNet® 2.0, in 2008. PermaNet® 2.0 is a polyester net coated with a deltamethrin-containing (55 mg/m²), wash-resistant resin. Both of these LLINs have shown high efficacy in laboratory studies and field evaluations in experimental huts (WHO 2001, 2008, Kulkarni 2006).

Olyset® (manufactured in 2008, serials and lots: 750274020106-8623BF16, 180219020105-8623BF16, 180219020122-8623BF16 and 590258020105-8623BF16) and PermaNet® 2.0 (manufactured in 07/2009 and all belong to the serial SPO9600) were tested under laboratory conditions in the laboratories of vector control (Biosafety Level 2+ Criteria, 26ºC ± 2 and 75% relative humidity) at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) (International Centre of Training and Medical Research) in Cali, Colombia.

Insecticide susceptibility - Before the evaluation, the *An. albimanus* strain from the CIDEIM colony, which was established in 2008 from a susceptible colony that belongs to the Universidad del Valle in Cali, was evaluated for insecticide susceptibility to different insecticides used for vector control in Colombia. Susceptibility tests were performed using impregnated bottles assay of Centers for Disease Control and Prevention (CDC) (Brogdon & McAllister 1998) with the diagnostic dose and resistant thresholds (cut-off time) established for *An. albimanus*: deltamethrin (12,5 µg - 30 min), lambda-cyhalothrin (12.5 µg - 30 min), dichloro diphenyl trichloroethane (DDT) (50 µg - 30 min) and malathion (50 µg - 30 min) (Fonseca et al. 2010). Twenty non-blood-fed females were introduced into 250-mL glass bottles coated internally with the diagnostic dose of the respective insecticide. Each test consisted of four impregnated bottles and one control bottle that contained only ethanol. At least three replicates were conducted for each insecticide. The mortality in bottle assays was evaluated every 15 min.

Biological assays - Regeneration time, heat-assisted regeneration bioassays and wash-resistance bioassays were evaluated following the methodologies proposed by the WHO (2005). For the bioassays, four sub-samples measuring 25 x 25 cm² were cut from each of the four nets from each net type. One piece was used for the baseline, two pieces were used for the regeneration time study (1 piece for 1 wash and 1 piece for 3 washes) and one piece was used for the wash-resistance study. For the negative control, one sample from a polyester net without insecticide was evaluated alongside the assays.

All bioassays followed the same protocol based on the standard WHO cone technique (WHO 2005) and using the insecticide susceptible *An. albimanus* colony from CIDEIM (tested before the bioassays). Each cone was placed horizontally on the net, and five adult females (non-blood-fed) of two-five days of age were exposed to each piece for 3 min. The mosquitoes were then removed from the cones and placed in resting tubes with access to water and sugar. The percentage of knockdown (KD) was measured every 10 min for up to 60 min after exposure and effective mortality was assessed 24 h after exposure. Each piece was tested 10 times using five mosquitoes per cone (50 mosquitoes per net) and samples were carried out on four pieces (0 washes, 1 wash, 3 washes and wash resistant) for a total of 200 mosquitoes. This bioassay was repeated in four different nets per each brand (800 mosquitoes total). The washing protocol was conducted as per WHO guidelines (2005).

Regeneration experiments - In order to determine the time period required for regeneration of the nets after standard washing and holding at 30ºC, bioassays were carried out at 24-h intervals on net samples washed and dried once and net samples washed and dried three times consecutively as described in the biological assays. Bioassays were conducted one day after the washes and continued until initial biological activity was restored.

Insecticide bioavailability (efficacy) curves were established and compared for nets washed once and three times consecutively. The time required (in days) to reach the plateau was the period required for regeneration of the net. If the two curves were different, the longer period was adopted as the washing interval to ensure that wash resistance was not overestimated. This time was used during the wash-resistance study.

Heat assisted regeneration bioassays - In order to determine whether biological activity of the Olyset® net could be restored by heat assisted regeneration, cone bioassays were conducted on net samples that were washed and dried once or three times and then heated for 4 h at 40ºC, 50ºC or 60ºC, compared to unheated nets (30ºC). The number of pieces per net, repetitions and number of mosquitoes evaluated during the bioassays were the same as those described in biological assays.

Wash resistance - Wash-resistance assays were conducted with the nets that recovered full insecticide efficacy over the testing duration. Regeneration was achieved by PermaNet® 2.0 but not by Olyset® LLINs. Resistance to washing was determined on net pieces washed at intervals required for regeneration, using the standard WHO (2005) wash, and dried and held at 30ºC. Bioassays were performed after zero, one, five, 10, 15 and 20 washes for a single piece of net from each of the four PermaNet® 2.0

(the same piece was washed consecutively and used in subsequent assays). The time (i) between each wash and (ii) between the final wash of the set and bioassays was determined by the regeneration time. Each bioassay was conducted just before the next wash.

Statistical analysis - Regression curves were drawn using, respectively, percentage mortality and KD vs. number of washes. Error bars in Figs 2-4 correspond to 95% confidence intervals. The Kruskall-Wallis statistic was used for the analysis. If the percentage of mortality in the negative control was above 5%, then a correction was made using Abbot's formula (Abbot 1987). The maximum number of washes providing mortality and/or KD above the cut-off point (≥ 80% mortality after 24 h and/or ≥ 95% KD after 60 min post-exposure) was reported.

RESULTS

Insecticide susceptibility - *An. albimanus* from the CIDEIM colony were tested for susceptibility to some of the insecticides used in Colombia. Fig. 1 shows the mortality of mosquitoes throughout the test. The colony of *An. albimanus* proved to be susceptible to all the insecticides tested, which included DDT, malathion, lambdacyhalothrin and deltamethrin.

PermaNet® 2.0 - Regeneration time - Average mortality and KD of mosquitoes on PermaNet® 2.0 were high after both one and three washes, as shown in Fig. 2. The percentage of mortality for the positive control (a in Fig. 2) was 100% during the three days of continuous bioassays. The nets that were washed one time (b in Fig. 2) showed a similar trend, with 100% mortality during the entire experiment. For the nets that were washed three times (c in Fig. 2), the percentage of mortality was > 98% (Fig. 2A). There was no significant difference between the nets (a-c). The percentage of KD for the three net types (a-c) was > 98% during the three days of continued bioassays (Fig. 2B). The percentage of mortality in the negative control during the three days of assays was < 5%.

Wash resistance - Mortality and KD were measured after one, five, 10, 15 and 20 washes with their corresponding unwashed control. Average mortality and KD of mosquitoes on PermaNet® 2.0 after the tested washes are shown in Fig. 3. After one and five washes, the percentage of mortality and KD (after 60 min of exposure) was 100% for both nets [control (a) and the 1 washed (d)]. At 10 washes, the percentage of mortality for the washed net (d) decreased to 96%, but the percentage of KD remained at 99%. After 15 washes, the percentage mortality for the washed net was 97%. After 20 washes, the efficacy of the net decreased to only 60% mortality and 80% KD. For the positive control (net unwashed), the percentage of mortality and KD remained high during the entire experiment (> 99%) (Fig. 3). The mortality percentage for the negative control was < 8% during the assays and the mortality percentage in the treatments was corrected by Abbott's formula.

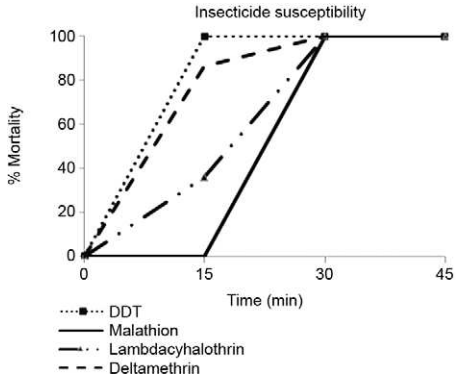

Fig. 1: percentage of mortality of *Anopheles albimanus* from Centro Internacional de Entrenamiento e Investigaciones Médicas colony, tested with Centers for Disease Control and Prevention bottle bioassays to determine the status of susceptibility to deltamethrin, lambdacyhalothrin, malathion and dichloro diphenyl trichloroethane (DDT).

Olyset® - *Regeneration time* - Evaluation with nets held at 30ºC, which represented ambient temperature, showed mortalities ranging from 70-100% in nets washed one time with KD rates of 80-100%. However, for the nets washed three times, mortalities were lower

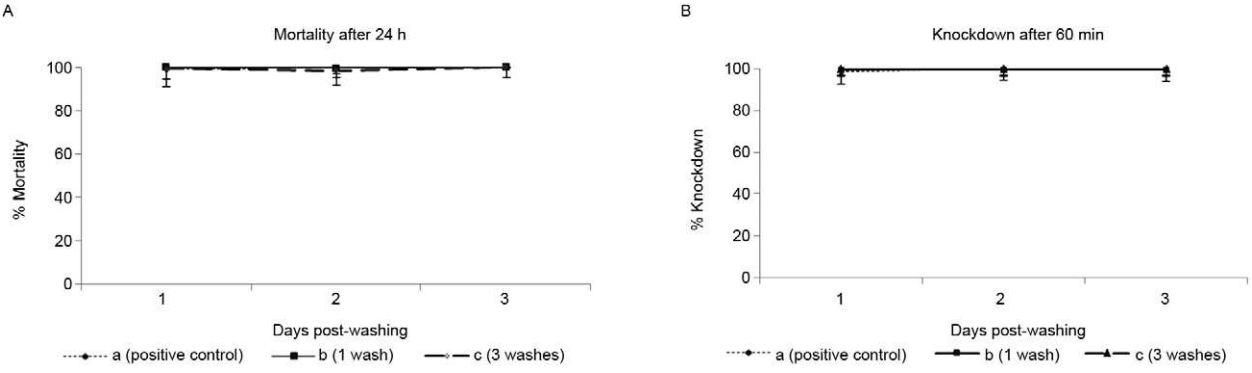

Fig. 2: percentage of (A) mortality 24 h and (B) knockdown 60 min post exposure in a 3 min World Health Organization cone test for PermaNet® 2.0. Three nets were used for the bioassays: a: positive control (unwashed net); b: net with one wash; c: net with three consecutive washes.

than 50% during the first eight days and started to increase with high variability during the following days. KD in the three-time washed nets recovered after eight days, though there was subsequent high variability and no plateau was reached (Fig. 4A).

Average mortality and KD of mosquitoes exposed to Olyset® nets held at 30ºC (ambient temperature), 40ºC, 50ºC and 60ºC are shown in Fig. 4. For the unwashed nets (positive control), the mortality percentage varied between 86-98%, even after 22 days of observations (Fig. 4).

For 40ºC and 60ºC, the mortality percentage of the nets washed once was quite similar to the positive control, but for 50ºC, the mortality was < 50% in the first day, increasing to 88% after the third day of observation.

None of the nets that were washed three times and treated with heat at any of the temperatures tested showed a mortality percentage > 70%. For samples heated to 40ºC, mortality remained at an average of 30% during the first three days of observation; for 50ºC, the mortality on the first day was only 10% and increased to only 66% by seven days; for 60ºC, mortality remained around 50% for 22 days. After seven days of evaluation, at 40ºC and 50ºC, the mortality percentage increased slightly but was not enough to reach the bioefficacy of the unwashed net (Fig. 4). For 60ºC, the mortality percentage was never higher than 60% during the 22 days of observation (Fig. 4C).

The KD percentage for the unwashed net (positive control) was similar to the mortality records in all the temperatures evaluated. The KD at 60 min varied from 80-100% during all the days of observation. The KD percentage for the nets washed once at 40ºC and 60ºC was high (> 80%) and was similar to that observed for the positive control. At 50ºC, the KD percentage was lower during the first two days, but increased by the third day to more than 80%. However, none of the nets washed once reached the cut-off point for KD (95%) (Fig. 4). For the three heated temperatures, the nets that were washed three times showed a very low percentage of KD that was not sufficient to reach the KD percentage of the nets that were washed once or the unwashed nets, even after 22 days of observation (Fig. 4).

DISCUSSION

The current study evaluated the regeneration time and wash resistance of two LLINs, PermaNet® 2.0 and Olyset®, under laboratory conditions.

The regeneration evaluation of PermaNet® 2.0 showed that total efficacy was recovered by 24 h after one and three washes. Mortality and KD were higher than the cut-off established for LLINs. These results allow us to evaluate wash resistance in this LLIN and showed that the regeneration time persisted for 15 washes without losing efficacy, although this decreased somewhat by 20 washes. This is slightly different than results from several other studies, in which the efficacy of PermaNet® 2.0 was maintained even after 20 washes under laboratory and field conditions against some of the most important disease vectors around the world (Kroeger et al. 2004, Gimnig et al. 2005, Graham et al. 2005, Sreehari et al. 2009, Atieli et al. 2010). This could be due to differences in insecticide susceptibility or landing behaviour of the CIDIEM *An. albimanus* colony compared to the other species evaluated. The efficacy of this net has also been shown to be very high even when undergoing different types of washing. Sreehari et al. (2009) observed increased efficacy of PermaNet® in producing > 80% mortality in *Anopheles culicifacies* and *Anopheles stephensi* mosquitoes after up to 20 hand washes and up to 10 machine washes. Atieli et al. (2010) concluded that PermaNet® 2.0 retained its efficacy longer with successive washes using three different washing methods (machine, hand and washing on rocks) compared to the three other brands of LLINs evaluated.

For the regeneration study of Olyset® nets, we evaluated the effect of different temperatures on regeneration time. Three different temperatures were evaluated (40ºC, 50ºC and 60ºC) in comparison to unheated nets (held at 30ºC). Although an increase in mortality was observed in all the heat-assisted assays over time, none of the samples washed three times and held at the three temperatures tested were able to recover baseline efficacy after seven days of evaluation or even 22 days after washing. The lack of determination of regeneration time, at either ambient temperature or following heating,

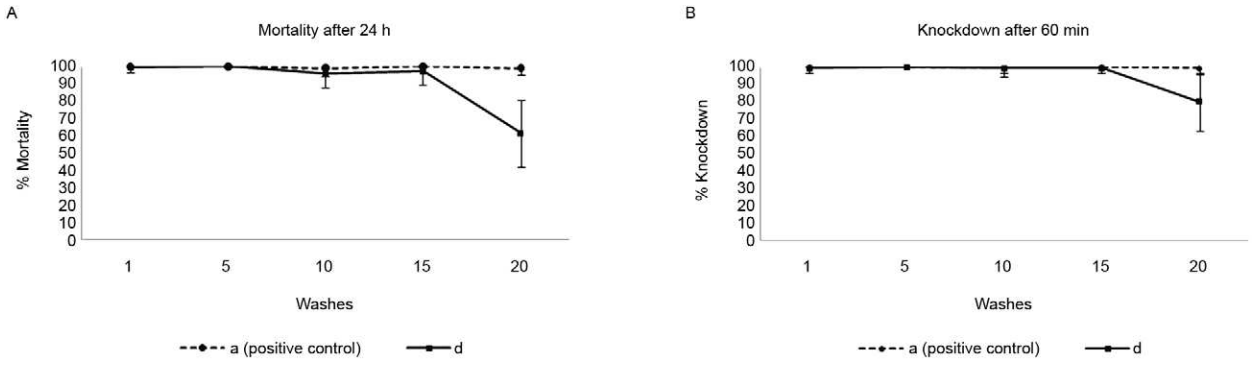

Fig. 3: percentage of (A) mortality 24 h and (B) knockdown 60 min post exposure in a 3 min World Health Organization cone test for PermaNet® 2.0 after one, five, 10, 15 and 20 washes. Two nets were used for the bioassays: a: positive control (unwashed net); d: net washed continuously.

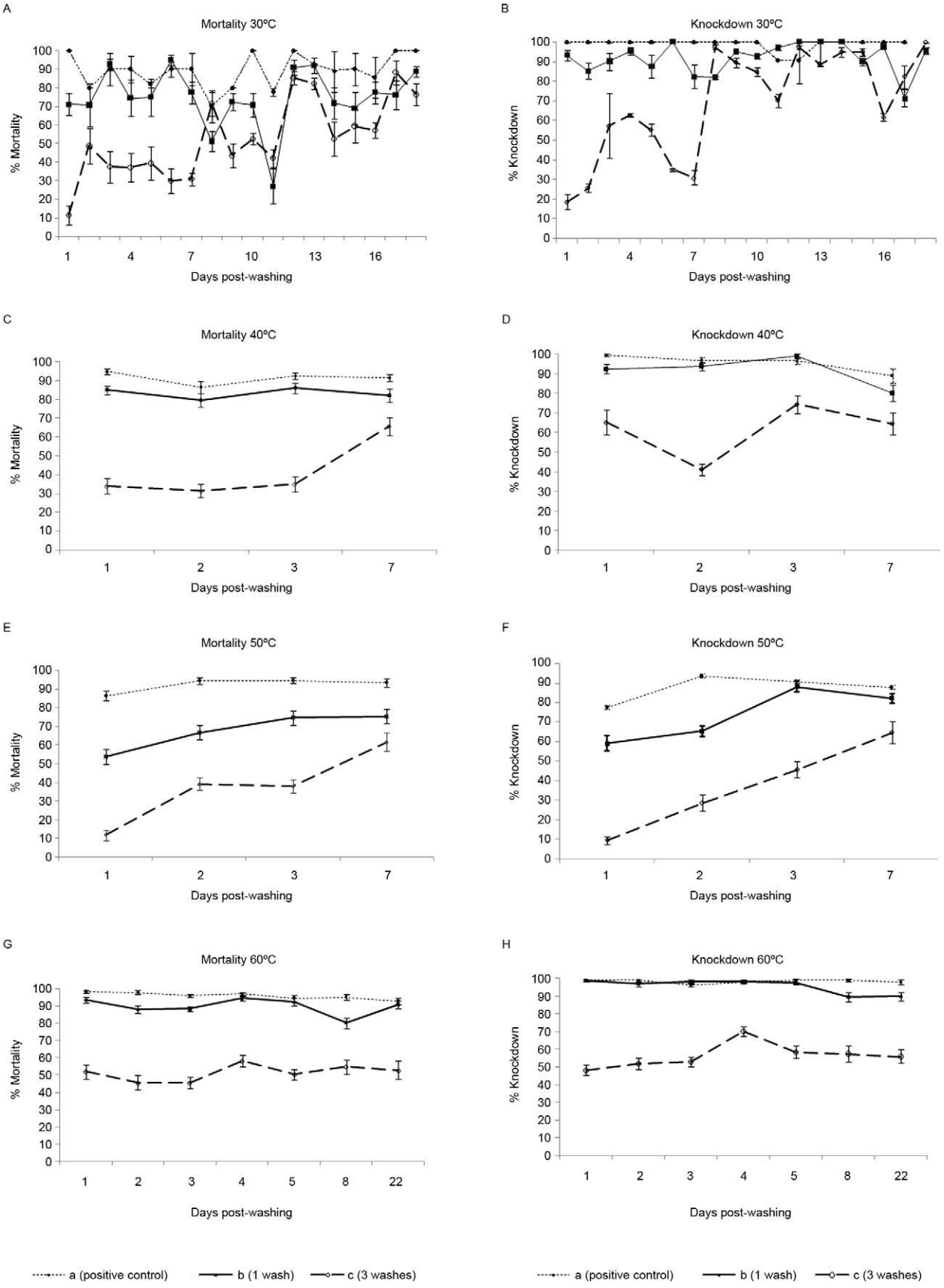

Fig. 4: percentage of mortality and knockdown for Olyset® nets washed one and three times. Regeneration study with temperature held at 30ºC (A, B). Heat assisted regeneration at 40ºC (C, D), at 50ºC (E, F) and at 60ºC (G, H). Three nets were used for the bioassays: a: positive control (unwashed net); b: net with one wash; c: net with three consecutive washes.

prevented us from completing the wash-resistance study on this net. This may also have been the case for other investigators, and WHOPES has indicated that "it is still not known how quickly regeneration occurs at ambient tropical temperatures" and that further studies on regeneration of Olyset® are needed (WHO 2005).

Our results on heat-assisted regeneration contrast with those obtained by other authors showing that a total recovery of the bioefficacy was reached over time or after heat-assisted regeneration. Olyset® nets are known to have a slow recovery bioefficacy under laboratory conditions, although a facilitation of its biological activity is expected by heating at 60ºC (Hougard et al. 2000). These results prompted the manufacturer to recommend exposing the net to direct sunlight to recover its efficacy faster; this accelerated diffusion due to heat exposure was also confirmed by Gimnig et al. (2005) and Sreehari et al. (2009). However, the knowledge that direct sunlight is harmful to pyrethroid-based insecticides, because ultraviolet rays break down pyrethroid molecules rendering the insecticide ineffective, prompted the WHO to recommend placing Olyset® in sealed polythene bags before leaving them for a few hours in the sun after washing (WHO 2003). A recent field study comparing the effectiveness of four different net brands showed that the Olyset® net retained more insecticide when dried under the shade (Atieli et al. 2010).

It is possible that observed differences in the regeneration of PermaNet® vs. Olyset® may be due not only to differences in insecticide bioavailability, but also to differences in the response of the test mosquito colony to the insecticides on the LLINs. It is known that the repellent capacity of permethrin can affect results obtained with Olyset® in cone tests [I Takaaki comment on Gimnig et al. (2005)]. This repellence may decrease the time the mosquitoes are exposed to the net during the 3-min time period, due to resting on the cone rather than the LLIN surface. *An. albimanus* may also differ in susceptibility to the two insecticides (permethrin and deltamethrin), though this could not be determined from the CDC bottle assay results as these were not conducted with permethrin. The differences observed in this study support the idea that although a regeneration time of < 24 h has been established in multiple studies of PermaNet® 2.0, further studies on the regeneration of Olyset® LLNs are needed, especially under local operational conditions (WHO 2009).

The rapid regeneration of PermaNet® 2.0 nets is associated with the wash-resistance properties in the resin that is coated on the polymer fibres during manufacturing. The wash-resistance study showed that this coating protects the insecticide impregnation for at least 15 washes without losing efficacy. Conversely, the Olyset® net is not coated; rather, the insecticide is incorporated, which requires the insecticide to migrate to the outside through the polymer matrix. This may be the reason for the significantly slower regeneration time in association with the possible repellence capacity of this net. It is important when making control decisions on optimal LLINs to consider if there are implications for the protection of populations and mass effects of the LLINs after field washing for the period in which a sub-lethal dosage of insecticide is available. This becomes of particular importance to personal protection where the physical barrier may also be compromised, such as where holes are present that allow vectors to pass through.

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