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Rapid colorimetric field test to determine levels of deltamethrin on PermaNet® surfaces: association with mosquito bioactivity

Michael D. Green[1], Frances Atieli[2] and Martin Akogbeto[3]

1 *Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA USA*
2 *Kenya Medical Research Institute, Kisumu, Kenya*
3 *Center for Entomological Research, Cotonou, Benin*

Summary

OBJECTIVE To evaluate a simple and inexpensive colorimetric test to measure the amount of cyanopyrethroid insecticide residue from filter paper exposed to mosquito net surfaces.

METHOD The net sampling protocol and colorimetric test (NetTest) were evaluated for deltamethrin-impregnated PermaNet® 2.0 by comparison with high-performance liquid chromatographic assays and mosquito mortality (WHO Cone Test).

CONCLUSION The observed correlation between the amount of deltamethrin adsorbed onto the filter paper and the entire amount of deltamethrin per unit area of net material was good: 0.967, five assays. The relationship between the surface levels of deltamethrin determined by the colorimetric test and the 'gold standard' mosquito bioassay reveals a relatively accurate field test with a sensitivity of 91.4% and specificity if 85.4% (76 samplings from 19 nets).

keywords malaria, bednet, cyanopyrethroid, deltamethrin, field test

Introduction

Malaria morbidity and mortality in sub-Saharan Africa have been significantly reduced through the use of insecticide-treated mosquito nets (bednets) (Lengeler 2004), but exposure and repeated washing can result in a reduction of efficacy due to loss of insecticide (Gimnig *et al.* 2005). Improvements in insecticide application techniques have resulted in long-lasting insecticidal nets (LLINs) with very high wash resistance (Kroeger *et al.* 2004). Among available LLINs and conventionally treated nets, the factory-pretreated PermaNet brand of net proved most resistant to controlled laboratory washes; still killing 50% of mosquitoes (WHO Cone Test) after 20 washes (Gimnig *et al.* 2005). The actual useful lives of LLINs are estimates based on extrapolations from controlled laboratory washing experiments and a few field studies.

The useful life of an LLIN may vary considerably from region to region. Variations can arise from malaria seasonality. A net that is used year-round is likely to lose insecticide more rapidly due to handling and cleaning than a net that is used only seasonally. Bednet users in some areas may clean nets more often than others and methods for cleaning may vary. The malaria vector species and its resistance to insecticide can vary considerably from region to region. Therefore, the degree to which LLINs lose insecticide to become ineffective will likewise vary regionally. By measuring the rate of insecticide loss, an LLIN distribution program can determine the optimum frequency for retreatment/replacement of nets in its specific region, and thereby maximize the cost-effectiveness of the program. There has been little investigation on the rate of insecticide loss for different nets in different environments and on the factors important in determining this loss rate (Miller *et al.* 1995; N'Guessan *et al.* 2001; Ordóñez González *et al.* 2002; Graham *et al.* 2005; Lindblade *et al.* 2005). The lack of data is partly due to the requirement of expensive and sophisticated analytical techniques such as gas chromatography or high-performance liquid chromatography to measure insecticide levels in the net. These devices are often difficult to acquire, especially in areas where bednets are most used.

Simple colorimetric assays to test insecticide levels have been developed. For example, halogens present in permethrin and other pyrethroid insecticides can be detected by using the simple Beilstein test where a portion of a net is extracted with acetone and the dried residue burned using a Bunsen burner (Muller *et al.* 1994). Although the presence of permethrin resulted in a green flame, the test produced false positives in coloured nets (Drakeley *et al.* 1999).

A quantitative test for cyanopyrethroids on treated bednets was developed using the inhibitive properties of these insecticides on a colorimetric product yielded by the action glutathione transferase enzyme (Enayati *et al.* 2001). This test also required extraction of insecticide from the net using acetone. Acetone extractions of nets in the field are cumbersome and unsafe, and the availability and stability of proteins for enzyme assays under field conditions are not reliable. Also, there are no reliable methods to measure the amount of insecticide available to the mosquito relative to the amount of insecticide incorporated in the entire net material as measured by acetone extraction. Therefore, our objective was to develop and evaluate a simple, sensitive, and inexpensive field test to assay cyanopyrethroid insecticides on the surface of LLINs and compare levels with mosquito mortality using the WHO cone test.

Materials and methods

Chemical assay

The colorimetric assay is based on the release of CN from the decomposition of cyanopyrethroids exposed to alkaline conditions (Figure 1). Cyanopyrethroid insecticides containing ester groups such as deltamethrin hydrolyse under alkaline conditions to form dibromochysanthemic acid and phenoxybenzaldehyde cyanodrin (Shan & Hammock 2001). Subsequently, the hydrolysable nitrile group (–CN) on the cyanodrin is lost to yield cyanide ions and phenoxybenzaldehyde (Patil *et al.* 1992; Shan & Hammock 2001). Under the same alkaline conditions, the released cyanide reacts with *p*-nitrobenzaldehyde to yield a cyanohydrin, which subsequently reduces o-dinitrobenzene to yield an intensely coloured purple product (Guilbault & Kramer 1966).

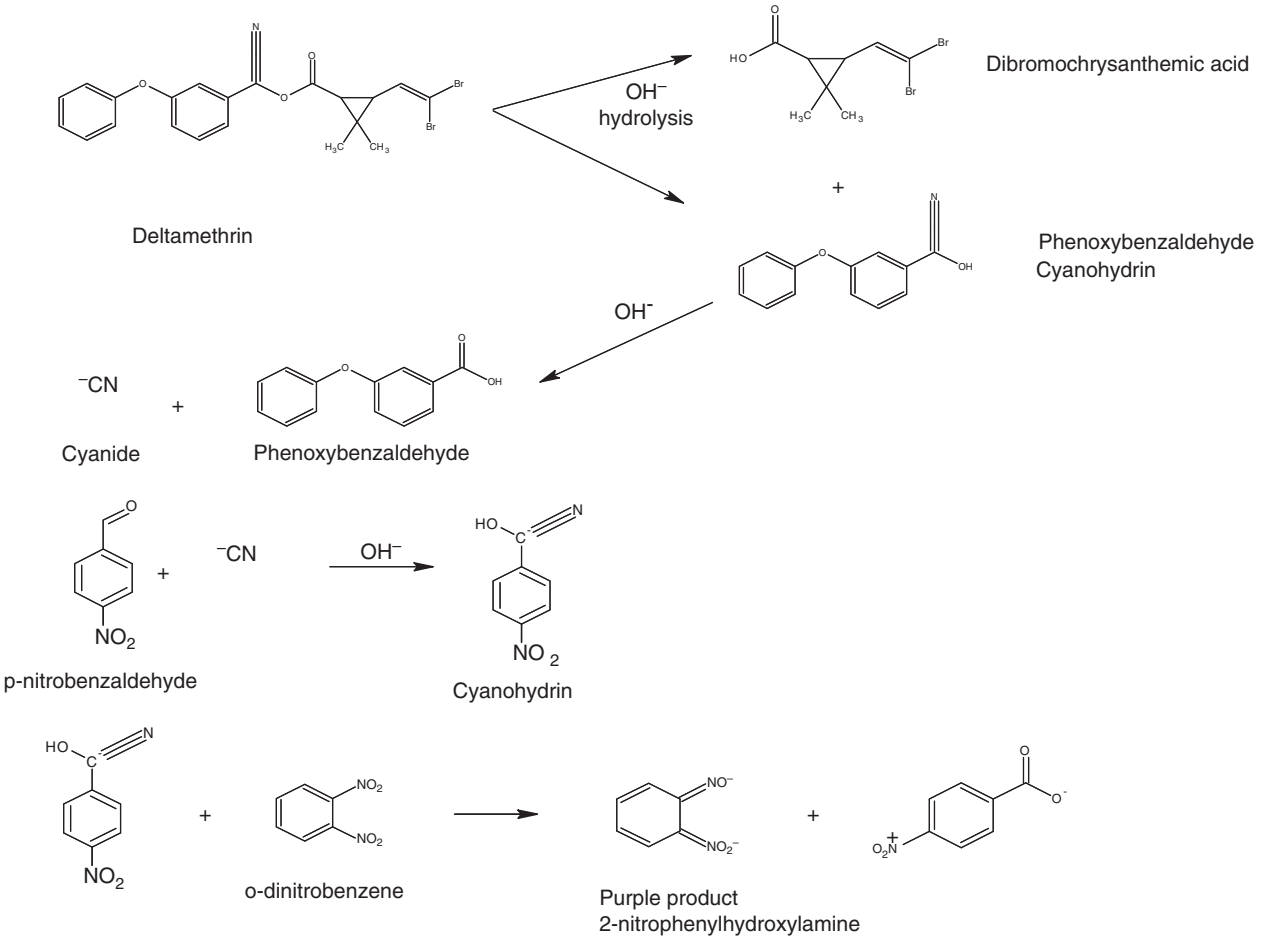

Figure 1 Schematic of chemical reactions involved in the colorimetric assay.

Reagent A consisted of *o*-dinitrobenzene (30.2 mg/ml) and *p*-nitrobenzaldehyde (27.2 mg/ml) dissolved in 80% methylcellosolve (20% water). Reagent B consisted of sodium hydroxide solution (16 mg/ml in 80% methylcellosolve). All these chemicals were purchased from Sigma-Aldrich Fine Chemicals, Milwaukee, WI, USA. Deltamethrin technical grade standard (99% purity) was purchased from Chem Services (West Chester, PA, USA). Two hundred microlitres of Reagent A was added to the wells containing sample and reference standard filter papers. After soaking for 5 min, 50 µl of Reagent B were added and the plates slightly agitated to mix the reagents. After a reaction time of 5–20 minutes, an intense purple colour formed in the presence of deltamethrin.

Net sampling method

A magnetic sampling device (MSD) was constructed by inserting and securing 4.7-mm diameter neodymium iron boron magnets (10 800 Gauss) (Radio Shack Corp., Fort Worth, TX, USA) into the cavities of two 1.5-ml Nunc tube caps. Whatman #1 filter paper discs (13 mm diameter) were attached to the ends of Nunc caps using a small piece of double-sided sticky tape (Figure 2). The sampled net (PermaNet® 2.0, Vestergaard-Frandsen, Denmark) was secured to a 9.4-cm inner diameter embroidery hoop and a sampling application guide was attached. The sampling application guide was constructed from a plastic CD where an oblong aperture 9.4 cm in length and 15 mm in width was cut. This device was used to guide the MSD across the net. The magnetic ends of the MSD were positioned within the application guide to both sides of the net and the MSD was manually slid back and forth 30 times (1 = back, 1 = forth). The surface area of the net (both sides) coming in contact with the discs is equivalent to 0.244 m^2. The magnets confer a consistent contact force between the filter paper and the surface of the net material; therefore direct pressure should be avoided by not pushing down onto the net with the MSD, but gently guided across the net. Samples were taken across the diameter of the hoop at two locations 90 degrees from each other. After sampling of the net, the two filter papers were carefully detached from the tape and deposited into the wells of a clear polystyrene 24-well tissue culture plate. Standard filter papers discs containing known amounts of deltamethrin (0, 0.10, 0.25, 0.50 and 1.0 µg) were prepared by adding specific volumes of a deltamethrin solution (in acetone) and allowing the solvent to dry. Two filter papers from each concentration were added to the wells to give final deltamethrin concentrations of 0, 0.20, 0.50, 1.0 and 2.0 µg per sample for the standard curve. Standard curve samples were used in every plate.

Measurement of colour intensity

The 24-well plate was placed upon a battery-operated light box (Visual Plus SV-650, Taiwan). A plastic hood was positioned over the light box and a digital camera (PowerShot SD750, Canon, Inc., Lake Success, NY, USA) placed in the hood orifice located 0.22 m above the plate (Figure 3). The camera was set for 'macro' and 'no flash'. This setup gave a consistent background illumination for each plate analysed. A digital picture of the plate was recorded and transferred to a computer where digital

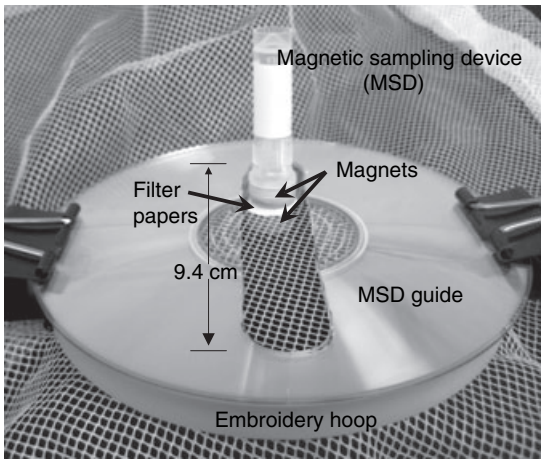

Figure 2 Design and use of magnetic sampling device to collect insecticide residue on net surface.

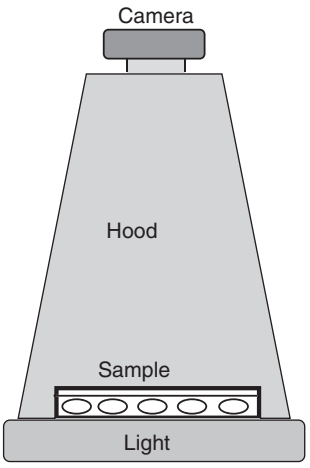

Figure 3 Setup for digital photography of sample plate.

image analysis software was used to measure colour pixel intensity (MVHimagePCv8, Global Systems Science, University of California; http://mvh.sr.unh.edu/software/software.htm).

The standard colour composites of a digital image on a computer screen consist of red, green, and blue pixels. The software measures the average frequency of pixels of a particular colour in a given area. The values are scaled from 0 to 100% intensity, with 100% representing the maximum colour present. If red, green, and blue are all present at 100%, then the resulting image will be colourless (white). Alternatively, if no coloured pixels are present the resulting image is black. Therefore, an inverse relationship exists between darkness of the image and the intensity of the mix of coloured pixels. A measurement of red pixel intensity (% RPI) as a function of deltamethrin concentration gave the most sensitive concentration curve (higher slope) from the purple product produced from the reaction.

The absorbance of the solution can be also measured spectrophotometrically using a wavelength of 520 nm. This technique usually requires a larger volume of reagents and may be impractical for a field test.

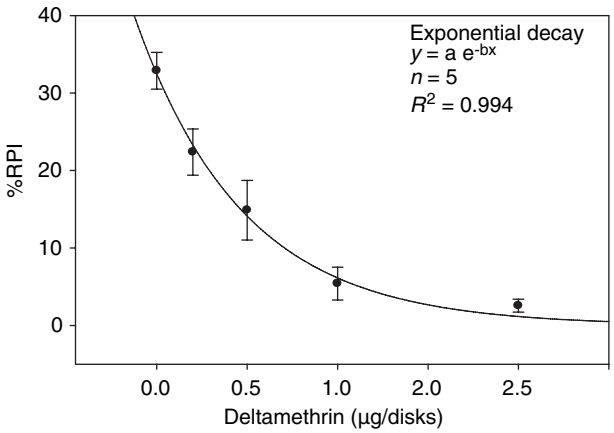

Figure 4 Relationship between % Red Pixel Intensity (%RPI) as measured using image analysis software *vs.* the amount of deltamethrin per disc. (●) Average of five samples ± SD.

Assay evaluation and correlation with net concentrations

All statistical analyses, i.e. Pearson Correlation coefficient, Receiver Operator Characersitic (ROC) curves, and *t*-tests were performed using MedCalc Vers. 9.6.0.0 (MedCalc Software, Belgium). Standard curve discs containing 0, 0.1, 0.25, 0.5 and 1.0 µg of deltamethrin were prepared by adding a specified volume of stock solution (in acetone) to the 13-mm diameter paper discs and allowing them to dry. Two standard discs were added to each well resulting in final deltamethrin amounts of 0, 0.2, 0.5, 1.0 and 2.0 µg per sample. The five colorimetric assays were performed as described above and the % RPI recorded for each sample. A plot of average % RPI *vs.* amount of deltamethrin per standard discs reveals the curve shown in Figure 4. An exponential decay formula ($y = a\ e^{-bx}$) was used to obtain the best curve fit for the %RPI *vs.* µg per sample relationship. Parameters '*a*' and '*b*' were determined using Sigmaplot Ver. 10.0 (Systat Software, Inc.) curve fitting program and standard curve sample concentrations recalculated to obtain percent accuracy and precision.

The amount of deltamethrin adsorbed onto filter paper was compared with the amount of deltamethrin per unit area of net using a high-performance liquid chromatographic (HPLC) method. PermaNets with various concentrations of deltamethrin were prepared by stripping the insecticide from the material using isopropanol solutions containing various amounts of water. A 21.6 × 27.9 cm piece of net was soaked in a flat pan containing 300 ml of 0%, 35%, 45%, 50%, 60% and 75% isopropanol in water. After 5 min, the sheet was removed, blotted to remove excess solution, and allowed to dry overnight. Deltamethrin extraction was accomplished by inserting an 8.3 × 8.3 cm piece of net into a glass vial containing 20 ml of acetone and 0.05 ml of λ-cyhalothrin (0.865 mg/ml) as an internal standard. After sonication for 30 min, 10 ml of solution was transferred to a polypropylene tube and dried with air. The residue was reconstituted in 0.5 ml HPLC mobile phase and 0.010 ml injected into the HPLC system. HPLC analysis was conducted using a 150 × 4.6 mm, C18 column with a mobile phase consisting of 80% acetonitrile, 20% water and 0.1% acetic acid. The flow rate was 1.2 ml/min with a column temperature of 30° C. The detection wavelength was 235 nm. This stripping method produced nets containing 54, 48, 31, 16, 2.9 and 1.7 mg/m^2 of deltamethrin with the respective isopropanol concentrations of 0%, 35%, 45%, 50%, 60% and 75%. Once the deltamethrin concentrations were established for each net sample, the colorimetric net test was performed. The results of the colorimetric test (µg/sampling) were compared with the net concentration of deltamethrin (mg/m^2) from which assay precision (% coefficient of variation), accuracy (% deviation of experimental amounts from actual amounts relative to actual amounts) and correlation coefficient were determined.

Mosquito bioassay comparison with colorimetric test

Mosquito bioassays as well as the colorimetric testing were performed on site at the Center for Entomological

Research (CREC), Cotonou, Benin. A kit containing all materials required for the colorimetric deltamethrin net assay was prepared at CDC and transported to the site. Chemicals and solvents were pre-measured into tightly sealed polypropylene bottles. The methylcellosolve used in the assay contained 20% water, rendering this solvent non-flammable for safe transport. The appropriate amount of solvent was added to the pre-measured reagents a few hours before beginning the assay to assure good dissolution.

Blue PermaNet 2.0 nets (mfg: 2–3/07; hung o/a 7–8/07) were collected from houses in Atlantique Province, Benin in July 2008 (approximately 10 months after they were distributed and hung). Mosquito bioassays were conducted using the WHO-approved cone test methodology (WHO 2005). Field-collected female mosquitoes, *Anopheles gambiae s.s.* AKRON strain, approximately 2–5 days old, were used in the bioassay. Four locations, i.e. top (A), side top right corner (B), side middle (C), and side bottom left corner (D) of the net were assessed (Figure 5). The same locations were sampled for the colorimetric test.

Results

Assay evaluation and correlation with net concentrations

Curve fitting using the exponential decay equation ($y = a\ e^{-bx}$) produced a good fit ($R^2 = 0.994$) in the range of 0.2–2.0 μg for deltamethrin/discs (Figure 4). These values were chosen to cover the range of deltamethrin amounts adsorbed onto the discs from nets containing up to 55 mg/m^2, the concentration that new PermaNets are stated to contain. Percent assay accuracy for five tests were 13%, –7%, 10% and –22% while assay precision was 23%, 18%, 15% and 24% for deltamethrin concentrations of 0.2, 0.5, 1.0 and 2.0 μg/filters, respectively. The limit of detection [mean of blank +6 × SD (Massart *et al.* 1988)] for surface levels of deltamethrin using the colorimetric assay is 0.3 μg/m^2. Using regression analysis from the plot in Figure 6, this is equivalent to a net containing 6 mg/m^2 of deltamethrin.

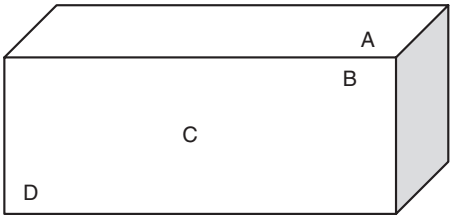

Figure 5 Sampling positions for a fully deployed bednet.

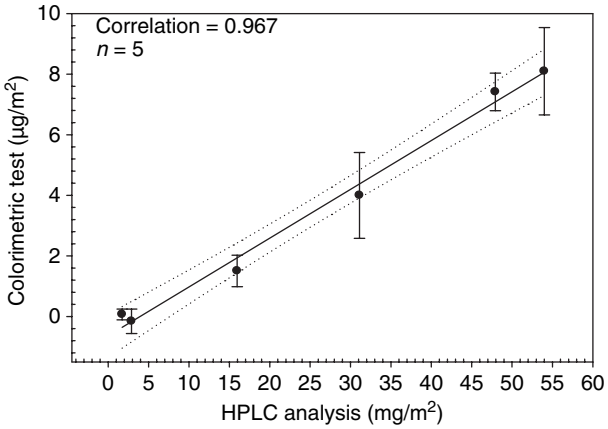

Figure 6 Comparsion of surface levels of deltamethrin (Colorimetric) with concentration of deltamethrin per unit area of fabric (HPLC). (●) Average of five samples ± SD; (·····) 95% CI.

Figure 6 shows a good correlation (0.967) between the amount of deltamethrin adsorbed onto the paper discs and the amount of deltamethrin per unit area of net material. The described methodology requires the 13-mm diameter disc to be rubbed across 9.4 cm of net material. Considering the amount of deltamethrin adhered to the disc and the surface area of contact (13 × 94 mm), about 0.013% of the total amount is rubbed away from the surface of the net material using the magnetic sampling device.

A net with mosquito bioactivity exhibiting mortality of 80% or less is considered by WHO as a failed net (WHO 2005). Using this criterion to categorize nets as pass or failed, a receiver operating characteristic (ROC) curve was used to determine optimal sensitivity, specificity, and the cutoff criterion for the assay. The area under the ROC curve (a measure of accuracy) is 0.876 (0.781–0.941, 95% CI, $P < 0.0001$; 1.0 = perfect test, 0.5 = worthless test), revealing the colorimetric test to accurately discriminate a failed or passed net based on mosquito mortality bioassays as the 'gold standard'. A significant positive correlation was observed (Corr = 0.637 (95% CI; 0.481–0.754) $n = 76$, $P < 0.0001$).

Figure 7 illustrates a dot histogram obtained from the ROC results. Using field-collected AKRON strain of *A. gambiae* mosquitoes with suspected resistance to deltamethrin, a net surface level of 2.8 μg/m^2 (95% CI 2.3–3.3) or below would indicate a failed net. This value is equivalent to a net with a total deltamethrin concentration of 21 (95% CI 18–23) mg/m^2 as determined from Figure 6. The relationship between the surface levels of deltamethrin determined by the colorimetric test and the bioassay reveals a relatively accurate field test with

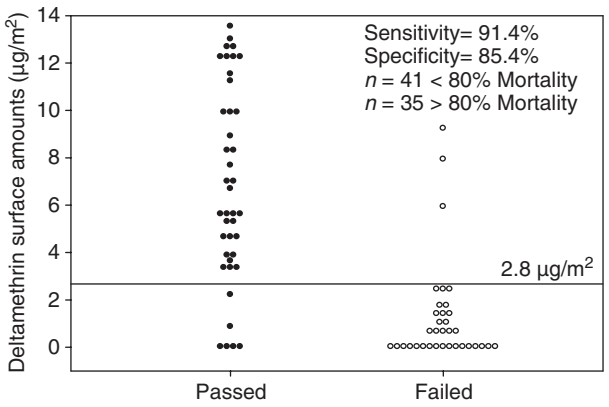

Figure 7 Dot histogram of deltamethrin amounts adhered to filter papers rubbed onto net surface. A net exhibiting 80% or less mosquito mortality is categorized as a failed net. The cutoff criterion of 2.8 µg/m^2 of deltamethrin was determined for optimum assay sensitivity and specificity using receiver operator characteristic curve (ROC).

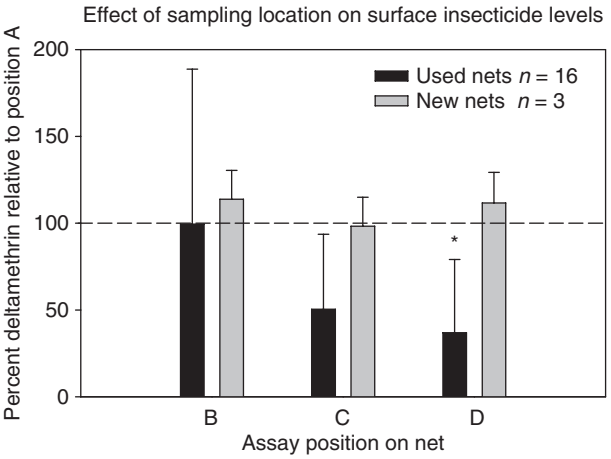

Figure 8 Effect of net sampling location on surface deltamethrin levels as controlled for washing (relative to position A). Position B = 99%, C = 50%, D = 37%. *P < 0.01 relative to same position on new nets.

minimal false positives (true positive rate or sensitivity = 91.4%) and false negatives (true negative rate or specificity = 85.4%) (Figure 7).

We compared deltamethrin surface levels for each sampling position of the net. Since the washing conditions for the nets were not known, values were calculated relative to the top position 'A' (Figure 5) of the net to control for insecticide loss due to washing. It is assumed that loss of insecticide at position 'A' due to contact with other surfaces such as skin, clothes, or bed linens is minimal. Figure 8 shows that only about 40% of the surface amount of insecticide is left at the bottom of the net (position D) relative to position A (top) and position B (top right corner). There were no observable differences in mosquito mortality at positions B, C, or D relative to position A.

Discussion

This highly sensitive colorimetric assay can detect residual deltamethrin adhered onto filter paper after exposure to impregnated net material. This reaction has been used to detect cyanide in blood, water, soil and industrial waste (Vesey *et al.* 1999; Favero & Tubino 2003). So far, the test has been demonstrated in our lab to work with deltamethrin, λ-cyhalothrin, and α-cypermethrin. Other cyanopyrethroid candidates for the colour reaction include acrinathin, cycloprothrin, β-cyfluthrin, ζ cypermethrin, β-cypermethrin, O-cypermethrin, cyphenothrin, esfenvalerate, fenpropathrin, fenvalerate, flucythrinate, flumethrin, τ-fluvalinate, tralomethrin and ZXI 8901.

Our simple collection technique provides a convenient, safe, and accurate way of assessing insecticide concentrations after washings and exposure to other surfaces. No flammable or toxic solvents are required, therefore the sampling may be conducted in the homes and the collected filter papers can be stored until the analysis can be performed. The use of the 24-well plate, along with image analysis results in minimal use of reagents. The digital photos can be stored and analysed at a latter date.

Figure 4 shows the plot of %RPI vs. µg/discs to be associated with exponential decay ($y = a\ e^{-bx}$) curve. This characteristic is likely due to colour saturation at the higher concentration. The reaction times depend on the ambient temperature and the quality of the reagents. Therefore, it is recommended that standard curve samples be used on each plate. Typically the reaction time should be no more than 30 min under ambient temperatures of 20–25 °C (warmer temperatures accelerate the reaction). New nets with higher concentrations of insecticides (55 mg/m^2) require only about 5 min to develop and can be used for rapid screening to assess the quality of new insecticide-treated nets. Assay sensitivity has been observed to decrease due to suspected degradation of reagents in solution; therefore, it is recommended that solution not be used after 2 days storage under tropical conditions.

The AKRON strain of *A. gambiae* was used in the mosquito bioassays (Cone Test). This strain is indigenous to this region of Africa and is suspected to have some resistance to deltamethrin. ROC analysis revealed a net surface concentration ≤ 2.8 µg/m^2 to be indicative of a

failed net (≤80% mortality). This value is equivalent to a PermaNet containing 21 mg/m^2 deltamethrin. In comparison, an average concentration of 18.5 mg/m^2 for nets (n = 4 PermaNet® 1.0) exhibiting mosquito mortality of 54.9% has been reported using *A. gambiae* (Kisumu strain) (Gimnig *et al.* 2005). Effectiveness of insecticide-treated nets relies on mosquito contact with surface levels of insecticide. Therefore, analysis of surface levels may give a better indication of net effectiveness relative to the total amount of insecticide present in the entire fabric. The described sampling protocol (rubbing the surface with filter paper) picks up approximately 0.013% of the total amount of deltamethrin per unit area. Therefore it is assumed handling and contact of the net surface with other fabrics (e.g. clothes, bed linens) and human skin as well as washing significantly reduces the amount of insecticide exposed on the net surface and subsequently affects mosquito mortality. When a net is fully deployed, as illustrated in Figure 5, lower portions of the net are more likely to be exposed to the floor, bedding material, and people. Figure 8 confirms this phenomenon where surface levels at the positions C and D are reduced relative to position A and B. New nets fresh from the package do not show this reduction of surface levels relative to position sampled. Although a significant positive correlation was observed between surface levels of deltamethrin and mosquito mortality, there was no significant reduction in mosquito mortality relative to sampling position on the nets. This may be a product of the variability inherent in typical bioassays. Also, many of the nets sampled were above the threshold value of 2.8 μg/m^2 exhibiting mosquito mortality of 100%.

The efficiency of insecticide adsorption onto filter paper from the net depends on the manufacturing technique used to make the LLIN. Some techniques use certain binders to attach the insecticide to the net material while other techniques incorporate the insecticide directly into the polymer by either mixing the insecticide and polymer prior to extrusion or using high pressure to force the compounds into the materials. We focused our evaluation on the popular PermaNet brand. Another limitation is that the colorimetric assay will not work with nets incorporated with insecticides not possessing a cyano group, e.g. permethrin. We are currently evaluating this technique for use with indoor residual spraying (IRS).

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Corresponding Author Michael Green, Centers for Disease Control and Prevention, Atlanta, GA, USA. E-mail: mdg4@cdc.gov