EVALUATION OF WASH RESISTANCE OF LONGLASTING INSECTICIDAL NETS, CURRENTLY AVAILABLE IN ETHIOPIA, AND COMPARISON WITH CONVENTIONALLY TREATED NETS.

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DEDICATION

This dissertation is dedicated to my parents and to my wife Lydia, who were always supporting me and have always been eager to see my success.
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ACRONYMS

ANOVA       Analysis of Variance
CTNs           Conventionally Treated Nets
DHS             Demographic and Health Survey
FMOH          Federal Ministry of Health
ITNs             Insecticide Treated Nets
KD               Knock Down
KT 50          Time to 50% Knockdown
LLINs          Long Lasting Insecticidal Nets
ORHB       Oromia Regional Health Bureau
RBM            Roll Back Malaria
TDR             Tropical Diseases Research
UNICEF       United Nations Children Emergency Fund
UV               Ultraviolet
WHO           World Health Organization
WHOPES   World Health Organization Pesticide Evaluation Scheme
Abstract

ITNs are widely promoted as means of preventing man-vector contact in the control of malaria. Development of Long Lasting Insecticidal Nets (LLINs) avoids the need for re-treatment which was a major operational problem faced by net owners. There are, however several factors which affect the efficacy of ITNs. Washing is one among the factors affecting the efficacy of ITNs by reducing the lethal concentration of insecticides on the net surface.

The objective of this study was to evaluate wash resistance of LLIN, currently available in Ethiopia, in two different washing procedures and compare with conventionally treated nets (CTNs) subject to repeated washes. Two types of LLINs: (1) Olyset® (2) PermaNet® and net treated conventionally with deltamethrin were exposed to two different washing procedures (washed on stone and basin). Then all nets were subject to mosquito bio-assay on WHO cone test. Mosquitoes were exposed to each net for three minutes. Knockdown rate was measured 60 minutes after exposure and mortality rate was measured 24 hr after exposure. Effect of sunlight on the efficacy of nets was also assessed.

LLINs performed well than CTNs in both washing procedures. PermaNet®, relatively, was the most wash resistant with 80% mosquito mortality in WHO cone bioassays after as many as 20 washes in nets washed on basin and 65% in nets washed on stone. Olyset® was also as wash resistant as PermaNet® with >75% mortality after 20 washes in nets washed on basin but when washed on stone, Olyset® performed less than PermaNet® with mosquito mortality 50% after 20 washes, P= 0.032. CTNs were the least wash resistant in both cases with 60% mosquito mortality in nets washed on basin and 33.3% in nets washed on stone just after 5 washes. Wash resistance of a net had significant difference when washed on stone and basin, P=0.031 by ANOVA. Nets dried in direct sunlight or in shade had no significant difference in mosquito mortality or knockdown rate, P= 0.602. In general, washing ITNs harshly rubbing on stone reduces effective life span of insecticides on nets so rapidly as compared to nets washed on basin. So washing nets gently should be recommended to keep LLINs effective throughout their physical life span.

Key words: LLINs, CTNs, washing procedures, wash resistance, efficacy.
1. INTRODUCTION

1.1. BACKGROUND

Malaria is a disease which can be transmitted to people of all ages. It is caused by parasites of the species *Plasmodium* that are spread from person to person through the bites of infected mosquitoes.

Malaria is clearly a global problem, approximately 40% of the world’s population, mostly those living in the world’s poorest countries, are at risk of malaria. Every year, more than 500 million people become severely ill with malaria. (Malaria site, 2005; Daniel E, 1999).

The global spread of malaria, although rarely publicized, continues to be the primary health and economic concern for over one-third of the world's population. In 1982, one million children under the age of five died as a direct result of malaria in tropical Africa alone (Malaria site, 2005). In the same year 150 million people fell victim to malaria parasite, resulting in 2 million deaths (Daniel E, 1999; Greenwood BM et al, 2005). Now, two decades later, those numbers are even higher. Each year more than 300 million acute cases are reported, unfortunately more people die from malaria today than 30 years ago (Malaria site, 2005; WHO, 2005). Estimates indicate that 1.5 to 2.7 million people die from malaria yearly, 70-90% of which are children under the age of five (Philips RS, 2001).

Malaria is Africa's leading cause of under-five mortality (20%) and constitutes 10% of the continent's overall disease burden (RBM, 2005). Around 90% of malaria deaths occur in Africa, mostly in young children. It accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits in areas with high malaria transmission (RBM, 2005). So too is the economic loss, which in Africa alone is estimated at more than 12 billion USD annually (Daniel E, 1999; Greenwood BM, 2005; Philips R.S, 2001; Krishna et al, 1997). It also has slowed economic growth in African countries by 1.3% per year, the compounded effects of which are a gross domestic product
level now up to 32% lower than it would have been had malaria been eradicated from Africa in 1960 (Heggenhougen et al, 2003).

In Ethiopia also malaria is the most important health problem where nearly 48 million (68%) of the total populations are at risk and three quarters (75%) of total land mass of the country is regarded as malarious (MOH, 2004). Every year, more than 4 million clinical cases are reported from health facilities and communities (MOH, 2004). Malaria is the leading cause of morbidity and mortality in Ethiopia. It accounts for 10 – 40 % of all outpatient consultations, 13 – 26 % of all inpatient admission and 13 – 35% mortality rates in various health facilities (MOH, 2004). It also contributes up to 20% of under-five deaths (UNICEF, 2006).

The burden of malaria control in the Twentieth Century had been largely taken up by World Health Organization (WHO), which adopted a formal policy on the control and eradication of the parasite in 1955. Between 1955 and 1969, WHO launched a series of campaigns to eradicate malaria by spraying homes with insecticide (Daniel E, 1999; Philips R.S, 2001). In some areas, this worked and halted malaria transmission. But in Latin America, Africa and most Asian countries, results varied and the disease persisted (Daniel E, 1999).

The hope of wiping out the disease finally was abandoned in 1969 and WHO officially declared the eradication program failure in 1976 (Adrienne M.L, 2005). WHO's goal was never achieved for a variety of reasons. First, the mosquitoes that transmit malaria rapidly developed resistance to the main insecticide being used, DDT. Secondly, resistance to chloroquine by strains of Plasmodium falciparum (Daniel E, 1999; Adrienne M.L, 2005)
The eradication program no longer exists, but has been replaced by the Roll Back Malaria program. The roll back malaria (RBM) program was launched in 1998 by WHO (Daniel E, 1999; RBM, 2004). The RBM Partnership’s goal is to halve the burden of malaria by 2010 (RBM, 2004). The strategy set to achieve this goal is to combine both prevention and cure. Those at risk must have access to the most effective preventive measures including vector control, and those suffering from malaria must have access to prompt and effective treatment (Daniel E, 1999; Adrienne M.L, 2005; RBM, 2004). The other two strategies of RBM include detecting early, containing or preventing epidemics, strengthening local capacities in basic and applied research (Malaria site, 2006).

There are different vector control methods. Currently available control measures include: Insecticide-treated nets, indoor residual spraying, space spraying, ultra low-volume sprays, larviciding of water surfaces, biological control, drainage, environmental sanitation and water management (WHO, 2006).

These measures have different effects. Some are for adult mosquito reduction. These include insecticide-treated nets, indoor residual spraying, space spraying, and ultra low-volume sprays (WHO, 2006). Peri-domestic sanitation, larviciding of water surfaces, intermittent irrigation, sluicing, biological control, drainage, environmental sanitation, and water management are helpful for larval control and breeding sites reduction (WHO, 2006).

Protection of the individual from mosquitoes can be achieved through a number of methods including mosquito repellents, aerosols, protective clothing, insect screens, setting of dwellings away from breeding areas, improvement of the design and construction of dwellings, and mosquito nets (Phillips R.S, 2001).
Mosquito nets are not a new idea in most malarious areas, but levels of net coverage vary enormously from place to place. Vigorous commercial net markets exist in many parts of Asia (Curtis CF, 1992).

Similarly, in some parts of Africa, net ownership was a well established social norm and nets were widely available and relatively cheap. For example in 1993, 62% of households in Dar es Salaam (Tanzania) owned a mosquito net (Kara H et al, 2004) and around 35% of households were found to own a net in urban Burkina Faso (Guiguemde TR, 1994). In other parts of Africa, by contrast, net ownership is a rare exception and nets can only be bought for high prices in the big cities. Most parts of Africa fall somewhere between these two extremes (Kara H et al, 2004).

In Ethiopia, historically there has been limited use of ITNs. The 2000 DHS found coverage rates for nets of 1.5% and for ITNs of 0.5% (Kassahun N et al, 2004). Distribution of ITNs through the health care delivery system was first introduced in returnee and resettlement sites in the Western part of the Tigray Region, in 1997 (FMOH, 2004). Since then scaling up of ITNs in the country has been done through various organizations (Kassahun N et al, 2004; FMOH, 2004). Ethiopia is now aiming 100% ITNs coverage to malaria risk areas at the end of 2007; with two ITNs per household (Rori N, 2005).
1.2. LITERATURE REVIEW

At present, the primary option in preventing malaria, at least until a suitable vaccine is found, is to break the chain of transmission between mosquito vector and human host. The WHO has adopted the use of ITNs as one of the main strategy for this in the roll back malaria program (RBM, 1999).

After the introduction of synthetic insecticides during World War II, it was soon realized that bed nets impregnated with insecticides might provide better protection against mosquitoes, and hence malaria infections, than untreated nets (Vector biology and control, 2006).

ITNs are considered one of the most effective prevention measures for malaria and, if properly used can reduce the risk of transmission by as much as 63% (WHO, 2006). ITNs have also been shown to reduce all-cause mortality among children < 5 years by approximately 20%. This translates to the prevention of almost 0.5 million deaths each year in Africa south of the Sahara (WHO/RBM, 2006).

There are limited data about household coverage with nets prior to the introduction of ITNs, but it is clear that the extent of net usage varied considerably. Pre-existing net usage was very high in some countries, such as the Gambia, and very low in others, such as Mozambique, with levels in most countries falling somewhere between these two extremes (WHO, 2003).

Although treatment on nettings with repellents and even DDT were tried to provide additional protection to individuals against blood sucking insects like mosquitoes, it was only recently appreciated that a net treated with insecticide offers much greater protection against malaria (Willem T, 2002).
The development of synthetic pyrethroids, in the 1970s, as a new class of highly potent insecticides with a relatively low toxicity for vertebrates and significantly fewer environmental effects compared with other classes of insecticides, caused renewed interest in the combined use of insecticides and bed nets for malaria control (Willem T, 2002).

Use of ITN easily wards off people from mosquito bites of endophilic and endophagic mosquitoes and its efficacy enhances when its use coincides with the biting rhythm of the mosquitoes and the sleeping time of the people who use them (RBM, 2004). There are also collateral benefits in providing overall personal protection from other domestic haematophagous insects (Willem T, 2002).

Insecticide-Treated Bed Nets (ITNs) also have at least three important purposes in the case of the infected person. 1- They protect the person from re-infection. 2- They prevent mosquitoes from biting the infected person and so reduce the chance that the parasite is passed onto other people, (an extremely important outcome). 3- They kill mosquitoes that land on or near the ITN, preventing other household members from being bitten (WHO, 1999).

Not only does the net act as a barrier to prevent mosquitoes biting, but also the insecticide repels, inhibits, or kills any mosquitoes attracted to feed. Thus ITNs provide protection both to individuals sleeping under them and to other family members. So the effect is so significant that use of ITNs is considered to be one of the most effective prevention measures for malaria (WHO, 2003).

Results on the efficacy of insecticide treated nettings (ITNs) were ably demonstrated by different studies. Randomized controlled trial studies, funded by WHO/TDR, were done in Gambia; Kilifi, Burkina Faso, and Kenya; Navrongo (Alonzo PL et al, 1991; d’Alessandro U et al, 1995; Habluetzel A
et al, 1997; Nevil CG et al, 1996). These studies demonstrated that ITNs could reduce all-cause child mortality by 16%-33% in children aged 6-59 months, and that they are a simple cost-effective public health intervention for malaria control.

Similar studies done in Cote d’Ivoire indicated that ITNs could reduce the prevalence of asymptomatic malaria infection by 12% and an estimated protective efficacy against malaria disease was 56% (M C Henry et al, 2005). A study done in Ghana found that the ITNs resulted a 17% reduction in all-cause mortality in children aged 6-59 months (F.N. Binka’J et al, 2002).

A study done in Asembo and Gem, Western Kenya, where malaria is transmitted throughout the year, All-cause mortality rates in infants (aged 1 to 11 months) were lower in villages with higher bed net use, compared with control villages. The number of Anopheles mosquitoes per house was reduced by 77% in houses with bed nets (Lindblade K A et al, 2004).

Other studies done in African settings of different transmission intensities have also shown that ITNs can reduce the number of under-5 deaths by around one-fifth; saving about 6 lives for every 1000 children aged 1–59 months. The incidence of clinical episodes of *Plasmodium falciparum* infection is reduced by 50% on average (WHO, 2003). ITNs are also efficacious in reducing maternal anemia, placental infection, and low birth weight when used by pregnant women (WHO/RBM, 2006; WHO, 2003; Lengler C et al, 1996).

Generally made of nylon, polyester, or cotton, these ITNs are most often treated with pyrethroids such as permethrin, deltamethrin and lambdacyhalothrin (RBM/ WHO, 2001). Treating the nets with insecticide provides double protection: not only does it safeguard the person (or people) inside the net, but if the person inside the net is suffering from malaria, by preventing mosquitoes access to the
gametocytes, so that they (the mosquitoes) cannot be infected, which limits the further spread of the disease (Greenwood B M, 2005; Lengler C, 1996).

ITN, at present, is available in two forms, one requires regular treatment with insecticides (conventionally treated nets) and the other is long-lasting insecticide treated nets (LLIN), which does not require any treatment throughout its life span (WHO, 2005). One important problem associated with the use of ITNs was the need for re-treatment after they have been distributed. Conventional ITNs require retreatment with insecticide every 6-12 months (Summary of eighth meeting, www.cartercenter.org). According to WHO, an LLIN should retain biological activity for at least 20 washes of standard WHO recommended protocol and should retain biological activity for at least three years of field use (WHO, 2005).

Evaluation of wash resistance and efficacy of LLIN comparing with conventionally treated nets has been conducted in different parts of the world, under laboratory, semi field and field conditions. The study done under laboratory condition to evaluate the wash resistance of four LLINs (PermaNet_ 1.0, Vestergaard- Frandsen, Denmark; Olyset_, Sumitomo Chemical Co., Japan), (Dawa_, Siamdutch Mosquito Netting Co., Thailand; Insector Athanor, France), and also a net treated with a process designed to increase its wash resistance. And compared them with conventionally treated nets (deltamethrin, 25 mg/m²) (Gimming JE et al, 2005).

All net types of the above study were washed using a WHO standard protocol and tested weekly using WHO cone bioassays with *Anopheles gambiae*. The permanent 1.0 was the most wash resistant with >50% mosquito mortality in WHO cone bioassays after as many as 20 washes. The Dawa net also retained some activity after repeated washing but exhibited wide variation in insecticide retention and biological activity after six washes as measured by 24 hours mortality of *A. gambiae* in WHO cone
tests. After 20 washes, all nets lost > 50% of their initial insecticide concentrations except for the Olyset net (Gimming JE et al, 2005).

Other study done in Colombia and Bolivia compared Wash resistance of PermaNet with that of conventionally treated nets under laboratory and semi-field conditions (Gonzalez JO et al, 2002). For the laboratory study, PermaNet was compared with nets treated with deltamethrin (K-O Tab® at 25 mg. /m²), lambdacyhalothrin micro-encapsulated suspension (15 mg /m²), and alpha-cypermethrin SC (40 mg /m²). Nets were washed gently for 5 minutes using bar soap and cold water. Bio assay test was carried out on each net after 3 washes while bio-assay test on PermaNet was done again after 4, 10, and 20 washes. After 3 washes, Anopheles mortality was 100% on all nets except for the net treated with lambda-cyhalothrin where mortality was 97.6% (Gonzalez JO et al, 2002).

Conventionally treated nets were not tested after 3 washes. Mortality on the PermaNet was 100% after 4 and 10 washes for both Anopheles and Culex species. After 20 washes, mortality was 81.3% and 87.5% respectively. For the study of wash-resistance under local conditions, women washed the nets in their usual way. Nets were soaked for 30-60 minutes in tap water with washing powder and then rubbed over rocks for 3-5 minutes. Nets were then allowed to dry in a shady place. When nets were washed every 2 days, vector mortality after 4 washes declined to 43.5% for deltamethrin tablets and 41.3% for deltamethrin SC. When nets were washed every 7 days, mortality after 4 washes was 43.3% for nets treated with deltamethrin, 63.8% for nets treated with alpha-cypermethrin, and 92.6% for the PermaNet. Mortality for the PermaNet was 83.7% after 10 washes (Gonzalez JO et al, 2002).

Bioassay tests were conducted in Islamic Republic of Iran against An. stephensi to measure the effects of washing, drying, dust, and smoke on the efficacy of PermaNet compared with a net treated with deltamethrin tablets at 25 mg /m² (Kayedi MH et al, 2002).
Time to 50% knockdown (KT50) increased from 529 seconds on an unwashed K-O Tab-treated net to 873 seconds after 15 washes. For the PermaNet, KT50 was 462 seconds on an unwashed net and 534 seconds on a net washed 15 times. The authors reported that the insecticidal activity of the PermaNet was significantly greater than the K-O Tab-treated nets after 5 and 15 washes. In addition, the bioactivity of K-O Tab-treated nets was significantly lower on nets washed 15 times, compared with nets that had been washed 5 times or had never been washed (Kayedi MH et al, 2002).

In a study done by Kate G. (Graham K et al, 2005) Anopheles mortality after contact with unwashed treated nets (the original version of PermaNet and two types of conventionally treated nets) was 100%. After repeated washing bioassays on white PermaNet gave 97.7–100% mortality showing no significant loss of insecticidal efficacy, whereas washed conventionally treated nets gave significantly lower mortality rates. Nets conventionally treated with alphacypermethrin also showed significantly reduced efficacy after washing: mortality ranged from 40.9 to 95.5% on nets treated with 20mg /m² after 8–21 washes and 49.1% on nets treated with 15mg /m² after 21 washes (Graham K et al, 2005).

WHO Pesticide Evaluation Scheme (WHOPES), did evaluation at LLIN in Montpellier, France; Duchon Herve, Hougard. In this evaluation using standard WHO cone test, there were no decline in knockdown caused by PermaNet® 2.0 even after 20 washes, while a significant reduction was observed after just 4 washes of conventional nets. Mortality decreased from 100% to 26% after just 2 washes of the conventional nets and fell below 10% after 6 washes. For the PermaNet® 2.0, a similar though much less marked trend was observed. Mortality remained stable at 30 – 50% after 10 washes (WHOPES 2003).
1.3. STATEMENT OF THE PROBLEM

ITNs are now widely promoted as means of preventing man vector contact in the control of malaria and form the cornerstone of malaria prevention within the recently launched Roll Back Malaria initiative (RBM, 2000). There are, however, several factors which affect the efficacy of insecticide treated materials; washing is being one among the factors that affect the efficacy of ITNs (Stewart T et al, WHO; Guiellet PF, WHO).

WHOPES evaluation of PermaNet under laboratory conditions concluded that PermaNet could resist up to 20 WHO standard wash under laboratory conditions (WHOPES 2003). This method comprises the use of de-ionized water and high quality soap (currently, “savon de Marseille” is recommended as the standard soap) (WHO, 2005) is completely different from our local situation.

Studies on washing ITNs gave different results in different countries (Gonzalez JO et al, 2002; Kayedi et al, 2002; Graham K et al, 2005). Different factors contribute for this varying result: the concentration of soap strongly affects the pH of the water, which may affect the rate of degradation of the insecticides. Water quality may also affect the rate of insecticide loss. Local washing procedures also vary from country to country which could also affect the rate of insecticide loss (WHO, 2005).

Studies done in other countries stated that local washing procedures like rubbing nets on stone has highly affected the efficacy of nets and shorten the expected life span of insecticides on the nets (Gonzalez JO et al, 2002).
The afore mentioned factors vary among countries and cultures and 80% of our population live in rural area and usually washes clothes on stones in rivers. So this study was aimed to find out if local washing practice has significant effect on the efficacy of ITNs by comparing nets washed on stone and nets washed on basin.

In general the current study

- Evaluate the efficacy of LLINs in local washing practices
- Is believed to give clue to health programmers to develop health education on ITN washing practices
- Can serve as a baseline reference for further research especially in field conditions.
2. OBJECTIVES

2.1. General objective:

To evaluate wash resistance of LLINs, currently available in Ethiopia, in two different washing procedures and compare with conventionally treated nets after different number of washing.

2.2. Specific objectives:

- To evaluate wash resistance of ITNs to determine the effect of repeated washing on their efficacy.
- To evaluate the effects of number of washing and direct sun light, on the efficacy of nets in each washing procedures on mortality and knockdown effect of *Anopheles arabiensis*.
- To compare the efficacy / wash resistance/ of LLINs and nets treated conventionally with K.O Tab (CTNS) after repeated washing on basin and stone.
3. MATERIALS AND METHOD.

A laboratory based experimental study was conducted to determine the efficacy of ITNs in two different washing procedures, using laboratory reared *Anopheles arabiensis* mosquitoes. The study was done in Nazareth insectory, 100 Km east of Addis Ababa. The insectory was established in 1961 (E.C) under Federal Ministry of Health (FMOH), Malaria control and prevention unit. But now the insectory is under Oromyia Regional Health Bureau (ORHB). The insectory rears *Anopheles arabiensis*. The insectory was used for this study purpose to get *Anopheles* mosquitoes which were not exposed to any insecticides, to avoid possibility of efficacy failure due to insecticide resistance. And also to use the laboratory facility for bioassay test.

3.1. Nets used for evaluation of wash resistance and efficacy test after number of washing

Three types of nets, 16 nets from each type (four replicates from each type) based on WHO recommendation (WHO, 2005) were tested (table 1). Among the three types of nets tested two were Long Lasting Insecticidal Nets (LLINs) and the other one was a net treated conventionally with K-O-Tab (CTN). The two long lasting nets were Olyset net from A-Z Tanzania, and PermaNet®, Vestergaard- Frandsen. For CTNs commercially available pre-treated nets were used.

The basic concept of the Olyset® net is that Permethrin is incorporated inside the raw polyethylene which is then extruded into a monofilament fiber from which the net is fabricated. The active ingredient is embodied within the fiber, from which it is slowly released over time to give the biological effect. Any insecticide that is used up or lost at the surface is constantly replenished by redistribution of the permethrin from within the fiber (Guillet PF, WHO).
PermaNet: is polyester net treated with 50-55 mg/m² deltamethrin. Insecticide is diluted in a wash resistant resin, which is coating the fibers (insecticide is generally mixed in a resin coating fibers). These nets can be washed many times, and will remain effective until insecticide concentrations fall below a critical threshold (Guiellet PF, WHO).

Table 1 Characteristics of nets used for evaluation of wash resistance

<table>
<thead>
<tr>
<th>Net type</th>
<th>Quantity</th>
<th>Type of Material</th>
<th>Insecticide</th>
<th>Insecticide Concentration (mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PermaNet</td>
<td>16</td>
<td>polyester</td>
<td>Deltamethrin</td>
<td>50</td>
</tr>
<tr>
<td>Olyset</td>
<td>16</td>
<td>Polyethylene</td>
<td>Permethrin</td>
<td>1000*</td>
</tr>
<tr>
<td>Conventional</td>
<td>16</td>
<td>Polyester</td>
<td>Deltamethrin</td>
<td>25</td>
</tr>
</tbody>
</table>

* Manufacturer gives the active ingredient as 2% Permethrin (w/w), which is approximately 1000mg/m².

3.2. Washing and drying procedures of nets used for Bioassay test

Bed nets were exposed to two different washing procedures: (1) on cemented washing area to simulate local washing procedure (2) on basin. From each net type, half of the nets, i.e.; eight nets were washed on basin, gently for about 5 minutes using tap water and bar soap. Among these eight nets four were dried under shade and the rest four were made to dry under direct sunlight. The other eight nets were washed by local women on a cemented washing area built for washing clothes, to simulate rubbing on rocks. Among these eight nets also, four were dried under shade and the other four were exposed to direct sunlight. (The woman was told not to wash too harsh nor less intensively but wash as she used to wash her own clothes). To assess the effect of sunlight, nets dried under shade and those exposed to
direct sunlight were compared on the base of mosquito mortality and knockdown rate in each washing procedures.

3.3. Bioassay tests on nets with *A. arabiensis* to evaluate wash resistance of nets based on mosquito mortality and knockdown rates.

A total of fifty two nets were subject to mosquito bioassay test, sixteen nets from each type, one PermaNet Control (unwashed throughout the study period), one Olyset control, one CTN control and one untreated control. Each net was subjected to a baseline bioassay. After the baseline bioassay, eight nets from each type were washed “under local washing procedures” and the other eight were washed on basin. The nets were washed at five days intervals and subsequent bioassays were carried out five days after the previous washing.

According to WHO pesticides evaluation scheme (WHOPES) WHO cone bioassays should be performed on nets washed 0, 1, 5, 10, 15 and 20 times or until the efficacy of the net falls below the WHO standard criteria for optimum efficacy of nets i.e., mortality $\geq 85\%$ and knock down rate $\geq 95\%$ to measure the efficacy of the insecticide treatment after repeated washing (WHO, 2003; Gonzalez JO, 2002).

All bioassays followed the same protocol. Three WHO plastic cones were affixed to each net and ten mosquitoes were introduced into each cone. Mosquitoes used were blood fed, 2-5 days old female *Anopheles arabiensis*. Thirty mosquitoes were exposed on each net for 3 min and then transferred to 1-pint paper cups. Knockdown was measured 60 minutes after exposure and effective mortality was measured 24 hour after exposure. Mosquitoes were considered knocked down or dead if they could not fly and could not stand upright on either the side or the bottom of the paper cups. One untreated control
net was also tested each day. Bioassays were done at temperature of 25 ±2°C and 70% Relative humidity (RH). Bioassay test was done at baseline, after 1st, 5th, 10th, 15th, and 20th washes for LLINs and at baseline, after 1st and 5th washes for nets treated conventionally with K-O-Tab.

3.4. Data Analysis:

Data was entered in to and analyzed using SPSS computer software. Knockdown and mortality rates, the effect of washing procedures, number of wash and effect of sun light on nets efficacy were compared among nets using ANOVA procedures. Effect of washing procedures, sun light and number of wash on each net type at each number of wash was also compared using Chi-squared test.
4. RESULTS

4.1. Effect of washing on efficacy of Nets

A total of fifty two nets were subject to mosquito bioassay test, sixteen nets from each type. Out of the sixteen nets eight were washed on stone and the other eight were washed on basin for each net type. Mosquito bioassay tests were done at base line, after 1\textsuperscript{st}, 5\textsuperscript{th}, 10\textsuperscript{th}, 15\textsuperscript{th}, and 20\textsuperscript{th} washes for LLINs and at baseline, after 1\textsuperscript{st} and 5\textsuperscript{th} washes for nets treated conventionally.

The twenty four hour mosquito mortality rate of all the three type bed nets at baseline was 100%. But as the number of washes increased, the efficacy of the nets decreased. This was true for all three net types at both washing procedures but the difference was more significant on nets washed on stone. Tables 2 and 3 show the effect of number of washes, washing type, and drying type on the efficacy of nets, mortality and knock down rate respectively.
Table 2. Percentages mortality rates of nets on *A. arabiensis* at different number of washing (pooled result).

<table>
<thead>
<tr>
<th>No. wash</th>
<th>Method of washing</th>
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<th>PermaNet %</th>
<th>Olyset %</th>
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Table 3. Percentages KD rates of nets on *A. arabiensis* at different number of washing (pooled result).

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<th>PermaNet %</th>
<th>Olyset %</th>
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At base line all three types of ITNs had similar mosquito mortality and knockdown rate. However it was found that as the number of wash increased the ability of ITNs to knock down or to kill mosquito decreased in all three types of nets (Fig 1). Additionally wash resistance, i.e. a tendency to remain effective under a number of washing, varied among net types, LLINs being more wash resistant than CTNs, p<0.001.

The efficacy of CTNs washed on basin fell below WHO standard criteria for optimum efficacy just after fifth wash (average mortality and KD 60%, and 80% respectively) figures 2 and 3.

But LLINs have shown excellent performance nearly with WHO standard criteria for optimum efficacy even after twentieth wash. PermaNet nets washed on basin had average mosquito mortality and knockdown rate of 98.6% and 100% respectively after fifth wash (figures 2 and 3). And Olyset Nets washed on basin had average mosquito mortality and knockdown rate of 96.6% and 100% after fifth wash (figures 2 and 3).

The difference between CTNs and long lasting nets washed on basin was significant. P=0.003 after first wash and P<0.0001 after fifth wash. But there was no statistically significant difference between the two long lasting nets washed on basin.
Figure 1. Average mortality rate of *A. arabiensis* on nets after number of washing.
When nets were washed on stone the difference among nets was high. Average mosquito mortality and knockdown rate of CTNs washed on stone after the first wash was 74.5% and 86.6% respectively (tables 2 and 3). Whereas average mosquito mortality and knockdown rate of Long lasting insecticide treated nets (LLINs) washed on stone after the first wash was 94.5%, 100% for PermaNet and 96.6%, 100% for Olyset respectively, P<0.001 (tables 2 and 3).

After the fifth wash average mosquito mortality and knockdown rate of CTNs washed on stone was 33.3% and 50%, Of Olyset was 78.8% and 93.3%, and of PermaNet was 86.6% and 96.6%. There was statistically significant difference between CTNs and Olyset, and CTNs and PermaNet, P<0.0001. But the difference between two LLINs at this point was not statistically significant. P=0.09.

As the number of washes increased the difference between two LLINs become high but it was not statistically significant. After the tenth wash average mosquito mortality and knockdown rate of PermaNet washed on stone was 78.8% and 90% respectively while of Olyset was 70% and 88.8% respectively. After fifteenth and twentieth wash average mosquito mortality and knockdown rate of Olyset washed on stone was 61.6%, 76.6% and 51.6%, 66.6 respectively. And of PermaNet was 73.3%, 84.5% and 64.5%, 73.3% respectively (tables 2 and 3).
Fig. 2. Bioassay mortality rate of *A. arabiensis* by net type and method of washing.
Figure 3. Bioassay KD rate of *A. arabiensis* by net type and method of washing.
4.2. Effect of Number of Washing on mosquito mortality and knockdown effects of the nets

Mortality and knockdown rate was assessed with the predictor variable of number of wash on each net. In this study it was recognized that number of wash was significantly associated with mosquito mortality and knockdown in WHO cone bioassay test. P< 0.001 by ANOVA. Though the difference between baseline bioassay test and bioassay after the first wash of LLINs was not statistically significant, statistically significant difference was observed on efficacy between unwashed CTNs, CTNs washed only once and CTNs washed five times (p< 0.0001).

In case of CTNs washed on stone the efficacy fell below the WHO standard criteria for optimum efficacy just after the first wash (mortality ≈ 74% and KD ≈ 86.6%). When compared to the unwashed net, the difference was statistically significant (p< 0.001). After the fifth wash the mortality and Knockdown rate of CTNs washed on stone fell to 33.3%, and 50% respectively (figures 2 and 3).

Number of wash also had significant association with mosquito mortality on LLINs. But the association level differed on nets washed on basin and nets washed on stone. On nets washed on stone, number of wash had strong negative association with mortality than nets washed on basin (figures 2 and 3).

In PermaNet nets washed on basin number of wash had lesser impact. After twentieth wash the mortality and the knockdown rate of the net was 84% and 96.6% respectively. This was above the WHO standard criteria for optimum efficacy. The difference between consecutive bioassay tests was also not significant.
In PermaNet nets washed on stone, as the number of wash increased the efficacy of the net decreased tremendously. After the tenth wash the net fell below the WHO standard criteria for optimum efficacy. The difference between every other bioassay tests was statistically significant. (P= 0.02)

Number of wash had similar effect on Olyset net too. As the number of wash increased the efficacy of the net decreased. The impact was higher on nets washed on stone (figures 2 and 3). Although repeated washing has reduced the efficacy of the net, on nets washed on basin, the difference between consecutive bioassay tests was not statistically significant.

In Olyset washed on stone, as the number of wash increased the mortality and knockdown rates decreased significantly. Mortality at baseline was 100% and mortality after fifth wash was 78.8% (p<0.01).
4.3. Effect of method of washing on mosquito mortality and knockdown effects of the nets

The effect of method of washing on nets was assessed by washing nets on basin and washing on stone. For each net type, nets were washed on basin and equal numbers of nets were washed on stone. Controlling for a net type, a net washed on basin and washed on stone resulted different mortality and knock down rate. (Tables 2 and 3).

For conventionally treated nets, nets washed on basin yield significantly higher mortality and knockdown rate on bioassay test after the first and fifth wash than nets washed on stone, P<0.001 (Fig. 2 and 3).

Wash type had similar effect on long lasting nets too. For PermaNet, nets washed on basin had more mosquito mortality and knock down rate than the nets washed on stone. The difference was more significant as the number of wash increased (fig 2 and 3). Just after the 10th wash PermaNet washed on stone fell below WHO standard criteria for optimum efficacy (mortality = 78%, KD =90%). But PermaNet washed on basin remained effective even after 20th wash (mortality = 84.45%, KD = 96.6%).

Olyset, as PermaNet and conventionally treated nets, shown significantly different results when washed on stone and on basin. The efficacy of Olyset net washed on stone decreased dramatically below the WHO standard criteria for optimum efficacy just after fifth wash (mortality = 78.8% and KD = 93.3%) (Fig.2 and 3). Where as Olyset net washed on basin was still effective even after 20 washes (mortality =80% and KD=90%).

In general it was seen that wash type was significantly associated with mosquito mortality and knockdown on nets P=0.001 by ANOVA.
4.4. Effect of the way nets were dried on mosquito mortality and KD of nets

Mortality and knockdown rate was also assessed with the way nets were dried. Nets washed under both conditions i.e.; stone and basin were accessed to dry under shade and direct sunlight. Half the number of nets washed on stone were dried under shade and the rest half were dried under direct sunlight after every wash. The same was done for nets washed on basin. Then bioassay test was done on each net, dried under shade and direct sunlight, to evaluate mosquito mortality and knockdown rate.

In this study it was seen that dry type has no significant association with mosquito mortality and knockdown rate on nets, P=0.921 by ANOVA. Chi-square test was also done for each type of net washed under both conditions, after each number of wash, on nets dried in shade and sun. The result has showed that there is no statistically significant association between dry type and mosquito mortality / knockdown rate of nets (Figures 4 and 5).
Figure 4. Effect of type of drying on mosquito mortality of nets washed on basin.
Figure 5. Effect of type of drying on mosquito mortality of nets washed on stone.
5. Discussion

The results in this study revealed that method of washing and number of wash were significantly associated with mosquito mortality and KD rate on nets, but dry type had no significant association with nets efficacy. In this study it was observed that nets washed on stone had lower mosquito mortality and KD rate than nets washed on basin for all three types of nets. It was also observed that as the number of washes increased, the efficacy of nets decreased for all three types of nets on both washing procedures, though the effect was very high on nets washed on stone; i.e. number of washes had strong negative association with nets washed on stone.

At the baseline, bioassay mortality rate was 100% to all three types of nets. But as the number of wash increased LLINs performed better than CTNs in both washing procedures.

Similar results to these findings were found in Colombia (d’ Alessandro U et al, 1995), Tanzania (Nevil CG et al, 1996), Islamic Republic of Iran (Habluetzel A et al, 1997), WHOPES evaluation in France (Lengler c et al, 1996). In Colombia wash resistance of PermaNet was compared with that of CTNs under laboratory and semi-field conditions. In both procedures PermaNet performed better than CTNs. In locally washed nets bioassay mortality rate of CTNs was 100% at baseline but declined to 43.5% (d’ Alessandro U et al, 1995) just after four washes, where as bioassay mortality rate of PermaNet , which were washed locally, after four was 92.6% (d’ Alessandro U et al, 1995).

In a study done in Islamic Republic of Iran, median knock down time for CTN was 873 seconds while median knock down time for PermaNet was 534 seconds after 15 washes (Habluetzel A et al, 1997). According to the authors of the study the insecticidal activity of PermaNet was significantly greater than the CTNs after five and fifteen washes (Habluetzel A et al, 1997).
In a study done by WHOPES in France no decline in knock down rate cased by PermaNet even after 20 washes (100%), while significant reduction was observed after just two washes of CTNs. Mortality decreased from 100% to 26% just after 2 washes, and fell below 10% after 6 washes (Lengler c et al, 1996).

Unlike LLINs where insecticide is diluted in a wash resistant resin, which is coating the fibers in case of PermaNet or where permethrin is incorporated within fiber and over time the insecticide migrates to the surface of the yarn to replace the one that has been removed by washing in the case of Olyset, washing could easily remove insecticide from nets that have been treated by conventional dipping.

In this study LLINs, washed in both methods, performed better than CTNs on WHO cone bioassay test P<0.05 by ANOVA. This could be because: 1- high insecticide dose on LLINs, 2- special care was taken to make LLINs wash resistant like coating the net fibers with wash resistance resin as in case of PermaNet or incorporating the insecticide within the fiber as in case of Olyset (Guiellet PF, WHO).

In this study it was seen that method of wash was significantly associated with net efficacy, P=0.001 by ANOVA. In CTN washed on stone mosquito mortality on unwashed nets and nets washed once is significantly different P<0.001. CTN washed on stone has resulted mosquito mortality rate much lower than the WHO standard criteria for optimum efficacy just after the first wash. Whereas CTN washed on basin had high mosquito mortality rate than washed on stone after the first and fifth washes.

Bioassay results of LLINs were also followed the same trend as that of CTN when washed on stone and on basin. In this study LLINs proved that they could resist up to 20 washes, if not washed harshly (not
rubbed on stone). Olyset and PermaNet washed on stone has failed below WHO criteria for optimum efficacy just after five and ten washes respectively, with mortality <80% and KD<95%.

In agreement with this study, studies done in Tanzania, Iran and Colombia reported that at baseline all net types had 100% bioassay mortality rate of mosquitoes. When CTNs exposed to local way of washing bioassay mortality rate of mosquitoes fell below 90% just after first wash. After fourth wash bioassay mortality rate of mosquitoes was 43.5% (d’ Alessandro U et al, 1995). Whereas CTNs washed under controlled conditions gently with water and a bar of soap had bioassay mortality rate > 90% even after third wash (Gimming JE et al, 2005; Kayedi MH et al, 2002; Lindblade KA et al, 2005).

In a study done in Colombia and Bolivia, PermaNet washed under laboratory condition has produced bioassay mortality rate of 100% after 10 washes while PermaNet washed under local condition has resulted bioassay mortality rate of 83.7% after 10 washes (d’ Alessandro U et al, 1995. The results indicate that washing ITNs harshly makes the ITNs lose the insecticide more readily than those washed on basin.

In this study no statistical difference, between PermaNet and Olyset nets washed on basin, was seen. Though there was little difference on bioassay result after fifth, tenth, fifteenth, and twentieth wash. Average mosquito mortality rate on PermaNet washed on basin after fifth, tenth, fifteenth, and twentieth wash was 98.8%, 91.65%, 86.6%, and 84.45% respectively. While that of Olyset was 96.6%, 90%, 84.45%, and 78.8% respectively. These differences were statistically insignificant, P>0.2.

In contrary to this study, other studies has shown that by six washes, under laboratory condition, mortality had declined to <10% for the Olyset net as that of the CTN and it was significantly lower than PermaNet 1.0, which was > 80% (Gimming JE et al, 2005). Studies done in Kenya, Colombia and
Bolivia, after 2 years of household use, also reported Olyset performed significantly lesser than PermaNet (Gonzalez JO et al, 2002; Lindblade KA et al, 2005).

The reasons for the differences are not clear. One possibility is that the mosquito strain used in the bioassays of the above mentioned studies may have developed some resistance to permethrin (Lindblade KA et al, 2005). The other possible alternative is that Olyset nets require heat assisted regeneration following washing to maintain their full biological activity against vector mosquitoes (Gimming JE et al, 2005). In the above mentioned studies nets were held at room temperature (22-25°C) for one week between washing. But the present study was done in Nazera where temperature was about 29-31°C at the study time.

However, studies which reported failure of Olyset nets just after few washes (Gimming JE et al, 2005; Lindblade KA et al, 2005) did a chemical analysis and reported that Olyset nets retained >80% of original insecticide dose, indicating that the failure of these nets was due not to a loss/lack of sufficient insecticide but rather to some problem with bio-availability of insecticide on the surface of the net fiber (Gimming JE et al, 2005; Lindblade KA et al, 2005).

Other studies done in Tanzania, in agreement with the present study, have shown Olyset nets which were used for four years were as effective as a new one (Maxwell CA et al, 2006). In a study done in Malaysia in laboratory to evaluate efficacy and wash resistance of Olyset Nets, the percentage mortality of mosquitoes exposed to Olyset net, after 15 washes with water only, was 95% (WHO, 2001). These results in agreement with the present study show that Olyset net washed gently is as effective as PermaNet.
This study has shown that LLINs could resist up to 20 washes if washed gently with bar soap and water. After twenty wash average mosquito mortality and KD rate was 84.45% and 96.6% respectively for PermaNet and 78.8% and 90% for Olyset. In contrary nets washed on stone resulted <80% mortality just after 10 wash in case of PermaNet and after 5 wash in case of Olyset.

Olyset nets washed on stone failed below WHO standard criteria for optimum efficacy just after fifth wash, average mosquito mortality rate of Olyset after fifth wash was 78.8%, while that of PermaNet was 86.6%. Though the difference was not statistically significant (P=0.141). The possible reason for this difference could be the harsh washing procedure removed much of bio-available insecticide from the surface of the net and the time between consecutive wash was not enough for full regeneration (Gimming JE et al, 2005).

The present study indicated that washing the nets harshly rubbing on stone removes the insecticides from the surface of nets so fast that it shortens the effective life span of the nets almost by half as compared to nets washed gently on basin.

The present study has also shown that drying nets, washed in either condition, in sun or shade had no significant effect among types of nets. Controlling for method and number of wash, nets dried in sun had no significant difference from nets dried under shade (P=0.921 by ANOVA)

In contrary to this study, a study done in Iran reported that there was significantly greater loss of activity in nets (PermaNet and CTN with K-O-Tab) exposed to sun (Kayedi MH et al, 2002). The possible reason for the difference could be: in the present study nets were exposed to sun light just for one day after washing, while in Iran’s study nets were exposed to sun for three days between washes. As pyrethroids are UV sensitive (NPTN, 2006), the prolonged exposure could make them lose their efficacy.
Limitations of the study

• Bioassay tests on conventionally treated nets were done at baseline, after first and fifth wash only, because of severe shortage of mosquitoes in the insectory.

• The study is limited to laboratory situation, which might not be completely similar to the actual situation.
6. Conclusion and Recommendations

6.1. Conclusion

There are several factors that affect the efficacy of ITNs. The present study demonstrated the effect of method of washing, number of washing and type of dry on the efficacy of ITNs against *A. arabiensis*. The study has shown that washing nets harshly on stone removes insecticides too fast from the surface of the net. It shortens the effective life span of the net almost by half as compared to nets washed gently on basin. LLINs washed on stone fallen below the WHO standard criteria for optimum efficacy just after tenth wash while LLINs washed on basin performed well even after twenty wash. The effect was so fast on nets treated conventionally. CTNs rubbed and washed on stone have fallen below WHO standard criteria for optimum efficacy just at the first wash.

The study also demonstrated that number of wash was negatively associated with nets efficacy; i.e. as the number of wash increased the efficacy of nets decreased. The study indicated that the effective life span of ITNs is determined how frequent the nets are washed and how are they washed.

In general:

- Frequent washing removes insecticides form the surface of ITNs hence decreases the efficacy
- Washing ITNs harshly on stone found to be important factor that decreases efficacy
- Though drying nets under shade is advisable as pyrethriods are UV sensitive, the study demonstrated that type of drying has no significant effect on ITNs efficacy.
6.2. Recommendations

- LLINs proved to perform better than CTNs in both washing procedures. So it is advisable to provide LLINs to the rural community as the CTNs require retreatment after 2-4 washes and the rural people do not have the tendency to get the nets retreated regularly.
- Washing LLINs harshly, rubbing on stone, makes the nets lose the insecticide at faster rate. So LLINs should be washed gently, without being socked for long periods of time as people usually do to their other clothes, to maintain their efficacy longer. It is advisable to use wash basin, if possible, otherwise it is better to use plastic sheets rather than washing and rubbing on stone, to clean ITNs.
- As Pyrethroids are UV sensitive, it is advisable to dry nets under shade.
- When nets are distributed to beneficiaries’, they should also be provided with information on how to and how frequent to wash.
- Further research is needed to evaluate the efficacy of nets in the actual field setting.
7. References

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Annex 1.

Format for recording results of susceptibility tests, on adult mosquitoes.

Investigator ______________________________

Name and address of institute ______________________________

Country ___________ Region __________ District ___________

Locality/village (for wild type) ____________

Species tested ________________________

Sex ________ Age /days/ ________

Physiological stage: blood fed□, non-blood fed□, Semi-gravid□, gravid□.

Sample source: Insectory reared □ wild type □

**Bed net Tested:**

PermaNet LLIN □ Olyset LLIN □ conventionally treated □

Control □

**Test conditions:**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Relative humidity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>From</td>
<td>From</td>
</tr>
<tr>
<td>Exposure period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holding period</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Test result *(For each single test)*

<table>
<thead>
<tr>
<th>ITN tested</th>
<th>No. mosquito Exposed</th>
<th>No. mosquito Exposed</th>
<th>Observed mortality (%)</th>
<th>Corrected mortality (%)</th>
<th>No. Knocked down</th>
<th>Knock down Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
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