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Evaluation of control of malaria mosquitoes (*Anopheles* spp.) using pine oil (Pinus sylvestris, family: Pinaceae) and Mygga (N,N-diethyl-3-methyl-benzamide)

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Abstract

Annually malaria is estimated to cause more than 1 million deaths, most of them occurring south of Sahara. Preventing transmission of malaria is therefore of great importance for both economic and public health reasons. Control of mosquitoes still much dependends on the use of synthetic insecticides, which sometimes cause serious environmental and health side-effects. Two benefits of using plant-derived chemicals for vector control are the reduced risk of inducing resistance and their usually lower risk to the environment. This study compares the repellency of two substances, pine oil and MyggA® (*N,N*-diethyl-3-methyl-benzamide) for the impregnation of mosquito bed nets. The concentration used was 0.5% for both subsatnces. Thirty bed nets were distributed, ten of each substance including unimpregnated (control) nets, to 30 houses in the village Antula, Guinea-Bissau, West Africa. Ten houses were sampled every morning six days a week between 20th September and 11th October 2007. No significant difference in mean number of mosquitoes recorded was found between the two substances used and the control, indicating that the concentration used was too low or that the substances are not effective for usage as mosquito repellents. However, control bed nets had a higher mean and standard deviation, though not significant, of mosquitoes recorded than pine oil- and MyggA®-impregnated nets, possibly indicating that the substances used had a weak repellent effect. Further studies using higher concentrations is warranted to evaluate pine oil and MyggA® for the impregnation of bed nets.

Key words: Malaria, bed nets, impregnation, MyggA®, pine oil

Introduction

Mosquitoes are the most important vectors of disease to humans. Malaria is spread to humans by *Anopheles* mosquitoes and is caused by parasitic protozoa of the genera *Plasmodium*, where *P. falciparum* and *P. vivax* are the two most common ones causing human malaria (Burkot and Graves, 2004). Globally, it is estimated that human malaria infects 402-515 million people and causes more than 1 million deaths each year (Korenromp, 2004; Snow *et al*., 2005). Around 60% of all cases and 80% of all deaths due to malaria occur in Africa south of Sahara, where the most severely affected groups are children and pregnant women (World Health Organization, 2003). Malaria also seriously affects the general state of health of people in malaria-endemic countries. For the countries that are most heavily affected, malaria is estimated to reduce the annual economic growth by 1.3% (Sachs, 2001).

Control of mosquitoes still very much depends on the usage of synthetic insecticides. They often have serious environmental side-effects, run the risk of inducing resistance to insects, are usually relatively toxic to humans and other vertebrates, and adversely affect the economies of Third World Nations. The benefits of using plant-derived substances for control of disease vectors are many. Numerous plants exploit the dependence of chemical cues for host-finding in herbivorous arthropods. Similar mechanisms for host-finding occur in mosquitoes and other blood-feeding ectoparasites. Thus, several plant species produce substances that act as toxins and/or repellents to arthropods. The risk of inducing resistance to the plant-derived toxins/repellents is less likely to evolve, which can be explained by the co-evolutionary history between plant-feeding arthropods and plants and also because the plant-derived toxic or repellent compounds occur in complex blends. Sometimes these compounds interact with synergistic effects. For example, *Hyptis suaveolens* (family: Lamiaceae) and *Corymbia citriodora* (family: Myrtaceae) are plants which contain substances known for their anti-arthropod properties (Jaenson *et al.,* 2006). *Hyptis* and *Corymbia* have been tested earlier by Pålsson and Jaenson (1999a), Pålsson and Jaenson (1999b) and Jaenson *et al*. (2008, in manuscript) in Guinea-Bissau. Both smouldering and fresh *Hyptis suaveolens* and *Corymbia* oil showed significant anti-mosquito effect.

The numbers of species worldwide are decreasing at an alarming rate. Estimates of these rates indicate that over the next 50 years, if nothing is done, 25% or more of all species will have gone extinct (Ehrlich and Wilson, 1991; Baillie and Groombridge, 1996). For plants as a whole, it is estimated that 20% of all species are threatened (Baillie *et al*., 2004). By not protecting our ecosystems and preventing loss of biodiversity, we are not only hindering the possibilities of finding new and effective medicines (Chivian, 2001), but are also hindering the eradication of poverty (EC *et al*.).

Guinea-Bissau is one of the smallest African nations located in the western parts of Africa, with borders to Senegal and Guinea, with a total population of ca. 1.2 million people. Guinea-Bissau is one of the least developed countries in the world, ranked 173 of 177, with a Human Development Index of 0.349 and a life expectancy of less than 45 years (UNDP, 2006). Each year in Guinea-Bissau about 60.000 new clinical cases of malaria are reported and about 800 deaths are caused by malaria. It has been estimated that about 200.000 people, or about 16.6% of the total population, are infected with malaria (WHO/AFRO, 2004), but this is probably a gross underestimate.

The target substances in the present study were pine oil (from *Pinus sylvestris*, family: Pinaceae) and MyggA® (containing 19% DEET, *N,N*-diethyl-3-methyl-benzamide). Pine oil has recently been tested against ticks (*I. ricinus*) with positive repellent properties (Pålsson, pers. comm., 2007). The active substance of MyggA®, DEET, has a well-known anti-mosquito effect (Fradin, 1998; Pålsson and Jaenson, 1999; Apelqvist, 2007). However, none of the substances has previously been tested for bed net impregnation.

Aim

The main aim of this project is to evaluate the potential of pine oil and MyggA® in the field as repellents on anti-mosquito bed nets against mosquitoes, particularly against malaria mosquitoes (*Anopheles* spp.), in Guinea-Bissau. The project focuses on these questions:

- Do the two substances actually repel the mosquitoes, and are the effects persistent for a long enough period and competitive with commercial products to be feasible for routine use for bed net impregnation (World Health Organization, 2002)?
- Do the substances work well enough to reduce the risk of malaria to the user (World Health Organization, 2002)?

Hypothesis

- The rooms of bed-nets impregnated with pine oil or MyggA® will have a lower density/occurrence of malaria mosquitoes compared to rooms with non-impregnated bed-nets.

Materials and Methods

Guinea-Bissau - Field

The project was carried out in collaboration with the Laboratório Nacional de Saúde Pública (LNSP) with Dr. Francisco Dias, Sr. Mario Gomes and other staff members of LNSP. The field experiment was carried out in the village Antula (Latitude: 11.896402; Longitude: -15.583184) between 20th September and 11th October 2007.

Before starting the field experiment, a village meeting was held explaining the purpose of the study and that all participation is voluntary. All participating households were registered by family name and house number. The most common composition of people sleeping under the bed nets were two adults and one child (< 16 years old).

In total, 30 bed nets of 20 m^2 were tested in the field. Ten bed nets were impregnated with the substance MyggA®, containing 19% DEET as the active ingredient, and ten bed nets were impregnated with the substance pine oil, respectively. The concentration used was 0.5% for each substance. The impregnated bed nets were tested together with ten untreated bed nets as control. The bed nets were randomly distributed to the 30 houses selected for the study, with one net per house.

Mosquitoes were collected using battery-operated aspirators and torches from Monday to Saturday between 06:40 and 08:30 in the mornings, both inside and on the outer surface of the bed nets. Ten houses were surveyed each day and all houses were visited two times a week. The mosquitoes were killed using chloroform, and stored in plastic vials until identified.

Four bed nets, two with MyggA® and two with pine oil, were excluded from the trial due to treatment errors during the impregnation process. Out of 190 sampling events performed 54 were excluded from the analysis. These sampling occasions were excluded due to errors during the standardised sampling procedure.

Mosquito identification

Identification of the mosquitoes collected was carried out using dissection microscope, where malaria mosquitoes, *Anopheles* spp., were separated from the other mosquito genera, and females from males. *Anopheles* mosquitoes were identified to species level (Gilles and De Meillon, 1968; Gilles and Coetzee, 1987) and other mosquitoes to genus level.

Statistics

Due to the high variation in the data, caused by large number of zero sampling events, or negative sampling, in combination with sampling occasions where many individuals were observed, the number of observed mosquitoes were logarithmically transformed to [log(x+1)] using Williams Mean, M_w, (Williams, 1937). This removes the dependence from variation on the mean, normalising the data, and allows for calculation based on data that has null values. To test the effect of using MyggA® and pine oil as anti-mosquito repellents on bed nets, the data of the number of observed mosquitoes was analyzed statistically using a 1-way ANOVA.

The number of collected mosquitoes was analyzed statistically using standard arithmetic values. Using a 1-way ANOVA, the differences in numbers between female malaria mosquitoes collected in pine oil and MyggA® impregnated bed nets and in untreated control nets was compared.

Results

A total of 281 mosquitoes were observed of which 73 were excluded from the analysis and a total of 228 mosquitoes were collected of which 50 were excluded from the analysis due to methodological issues, addressed in the discussion.

Mosquitoes observed

There was no significant difference between the number of mosquitoes observed (Table 1) between the two types of impregnated bed nets and the control (Pine oil: P>0.05, d.f. = 91, F-crit = 3.946; MyggA®: P>0.05, d.f. = 92, F-crit = 3.945). However, there was a slight difference in the mean ($M_{w)}$ number of mosquitoes, where the control nets had a higher mean ($M_{w)}$ and a higher standard deviation of mosquitoes compared to the impregnated bed nets (Table 1). Figure 1 shows the Williams mean number and standard deviation of mosquitoes observed on each sampling occasion for the different types of nets.

Table 1. Observed numbers of mosquitoes in Antula (N), Guinea-Bissau corrected with Williams mean ($M_{w)}$ and standard deviation (SD). Unimpregnated control bed nets are compared to impregnated bed nets (pine oil and MyggA®).

Test	N	M_w	SD	Sampling events	P
Control	90	2.86	2.76	49	
Pine oil	59	2.37	1.68	43	>0.05
MyggA	59	2.34	1.60	44	>0.05

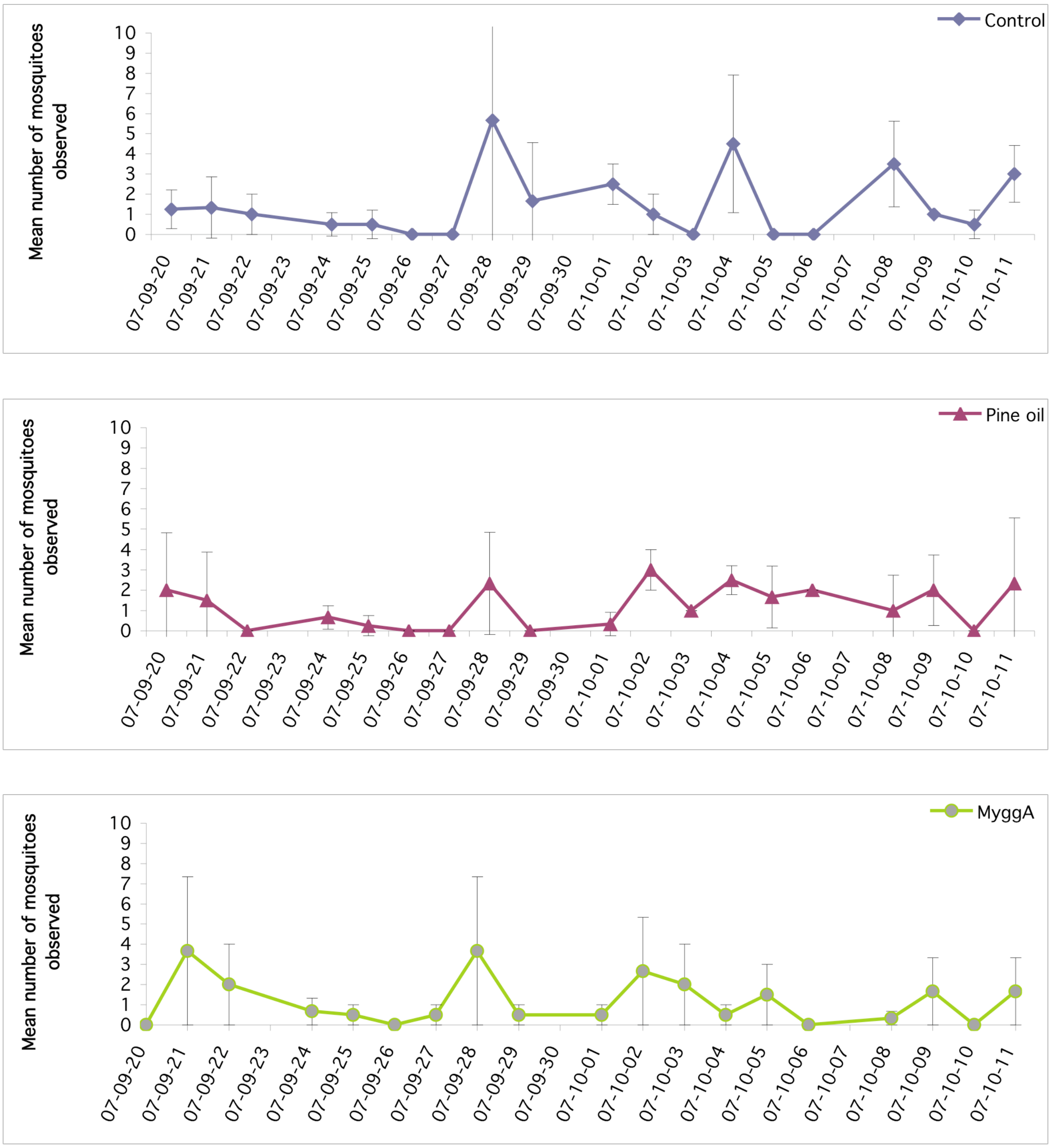

Figure 1. The mean number and standard deviation of mosquitoes observed on each sampling event for control, pine oil- and MyggA-impregnated bed nets between 20th September and 11th October 2007 in Antula, Guinea-Bissau.

Mosquitoes collected

Of the mosquitoes collected (Table 2), 176 were identified and 2 could not be identified. *Anopheles* mosquitoes composed 80.3% of all mosquitoes collected and *A. gambiae* (males included) composed 76.4% of all mosquitoes collected. The numbers of mosquitoes collected were similar between the different types of bed nets (60, 58 and 60 mosquitoes collected for control nets, pine oil-impregnated nets and MyggA-impregnated nets, respectively). The largest number of *Anopheles* mosquitoes was found in the MyggA impregnated bed nets and the lowest number was in pine oil impregnated bed nets.

No significant difference was found between the number of female malaria mosquitoes collected in pine oil and MyggA® impregnated bed nets compared to untreated control nets (Pine oil: P>0.05, d.f. = 14, F-crit = 4.667; MyggA: P>0.05, d.f. = 16, F-crit = 4.543).

Table 2. The total number and percentage of mosquitoes (N) collected between 20th September and 11th October 2007 in Antula, Guinea-Bissau. Control nets represent unimpregnated bed nets compared to bed nets impregnated with either pine oil or MyggA®.

		A. gambiae	***A. gambiae* male**	***A. funestus***	***A. squamosus***	***Aedes***	***Culex***	**Unident.**	**Total**
	N	38	2	3	3	6	7	1	60
Control	% of net	63.3	3.3	5	5	10	11.7	1.7	
	% of total	31.4	13.3	75	1.7	75	28	50	
	N	33	9	1	0	2	12	1	58
Pine oil	% of net	56.9	15.5	1.7	0	3.4	20.7	1.7	
	% of total	27.3	60	25	0	25	48	50	
	N	50	4	0	0	0	6	0	60
MyggA	% of net	83.3	6.7	0	0	0	10	0	
	% of total	41.3	26.7	0	0	0	24	0	
Total	**N**	**121**	**15**	**4**	**3**	**8**	**25**	**2**	**178**
	% of total	**68**	**8.4**	**2.2**	**1.7**	**4.5**	**14**	**1.1**	**100**

Discussion

This study found no significant difference in mean numbers of mosquitoes in relation to type of impregnated bed net (pine oil and MyggA®) compared to unimpregnated control nets. The hypothesis that pine oil and MyggA® would significantly repel mosquitoes, particularly malaria mosquitoes, was not confirmed. However, the control bed nets showed a slightly higher mean and standard deviation of the number of mosquitoes observed compared to the impregnated bed nets. This could indicate that the impregnation of bed nets with pine oil and MyggA® has a weak repellent effect.

A possible explanation to the negative results could be that the concentration used to impregnate the bed nets was too low to induce a distinct repellent effect against mosquitoes. MyggA® is mainly used as a roll-on repellent with a DEET concentration of 19% with a mosquito repellency of about 75% (Apelquist, 2007). Using the formula of Sharma and Ansari (1994) to calculate the percentage of repellency [(No. Control - No. Test Product) / No. Control] based on the M_w-values obtained in the present study, pine oil and MyggA® would have mean repellencies of 17% and 18%, respectively. With a concentration of 0.5%, these estimated repellencies would be fairly adequate for such a low dosage. This could possibly have been demonstrated with a larger sample size.

Biological control of malaria mosquitoes

The substances tested in this study have previously never been used for bed net impregnators. The active substance of MyggA®, DEET, has a known anti-mosquito effect (Fradin, 1998; Pålsson and Jaenson, 1999; Apelqvist, 2007) and is also effective against tick nymphs (*Ixodes ricinus*) (Jaenson *et al.* (2003) whereas pine oil has received no previous attention as a mosquito repellent. However, initial testing indicates that pine oil has some repellent effect against tick nymphs (*I. ricinus*) using a 0.5% concentration (Pålsson, pers. comm., 2007).

Using unimpregnated bed nets alone may possibly reduce the risk of contracting malaria, however using impregnated bed nets provides better protection. Bed nets treated with pyrethroids, such as permethrin, are effective and significantly reduce parasite prevalence in humans and densities of *Anopheles* mosquitoes resting indoors (Jaenson, 1994; D'Alessandro, 1995). There is evidence that the usage of pyrethroids to impregnate mosquito nets has selected for resistance in South African *A. funestus* (Brooke *et al*., 2001). Greenwood *et al*. (2005) propose that combining several insecticides when impregnating bed nets could prevent resistance. This, however, may not be very cost-effective which is one reason for the development of new, plant-derived chemicals. Takken (2002) argues that perhaps the best alternative would be the free distribution of unimpregnated bed nets, since many people fail to re-impregnate their bed nets which may also induce resistance quicker, in combination with early diagnosis and treatment.

Using an artemisinin-based combination therapy (ACT) together with insecticide treated bed nets, Bhattarai *et al*. (2007) studied the effect on the malaria burden in children under five and pregnant women in Zanzibar, East Africa. They found that by free distribution of ACT during 2002 - 2005 to all patients diagnosed with malaria the number of admissions due to malaria decreased with 77%, mortality of children with 52% and malaria attributed mortality of children with 75%. The total number of out-patient malaria diagnoses also decreased by 77%. The prevalence of *Plasmodium falciparum* was halved between 2003 and 2005 and following the free mass-distribution of insecticide impregnated bed nets in 2006 to children younger than five and pregnant women, prevalence of *P. falciparum* decreased another 10-fold.

Methodological issues

In the present study problems occurred that decreased the number of usable sampling events that could have been used in the analysis. Thus occasionally the inhabitants had fixed the openings of the bed nets or removed bed nets before sampling. For these reasons several sampling events had to be excluded from analysis. Also, on two occasions the bed nets, one control and one pine oil impregnated bed net, had been changed to an older version.

The strong smell of pine oil was a limiting factor for increasing the concentration when impregnating bed nets as pine oil has a strong odour that can cause discomfort for the people sleeping under bed nets. Eight days into the study one the inhabitants of one house removed their bed net, impregnated with 0.5% pine oil, due to complaints of the odour and replaced it with an older one. This may suggest that the active ingredient of pine oil-impregnated bed nets is still present after one week following impregnation.

Another important factor to consider is mosquito abundance at the time of the field study. This study suffered from relatively low mean number of mosquitoes observed and collected throughout the study period. The mean values are similar to those of Pålsson *et al*. (2004) during the lows of 1994 and 1995. If possible, prior to starting a field study, an initial estimation of the abundance of mosquitoes should be conducted.

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