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Development and Testing of Long-Lasting IRS Products While Revising the WHO Test Protocol

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Abstract

The paper describes the development of a long-lasting product for Intra-domicile residual spray (IRS) and shows it is possible to obtain a residual effect of nearly 2 years. However, to obtain that the methods currently recommended by WHO for laboratory evaluation had to be modified and approached methods closer to the semi-field and field evaluations as applied in later phases of WHO procedures. Surfaces with high pH resulted in short residual effect unless the formulations were mixed with a silicone coating. Screening in huts constructed for the purpose was realised by dividing the wall surfaces in 25 test plots of 0.5 m² where formulations were applied randomly with more repeats the closer to the final formulation. Mud and concrete surface were more challenging than wood surface and stones and these could be dropped for screening. Wall surfaces heated by sun were repellent to non-blood fed mosquitoes, and the test in huts were limited to the mornings. However, blood fed mosquitoes were not repelled. Cone tests on mud-walls are complicated by the uneven structure of the surface and a better way of attaching cones to avoid mortality errors was developed. Formulations that can be applied and last for two mosquito seasons produce big cost savings for IRS programs, since program costs are mostly application costs.

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Background

Malaria vector control relies primarily on two types of interventions: use of insecticidal bed nets, especially the Long-Lasting Insecticidal nets (LLIN) and the application of insecticides on wall, intra-domicile residual spray (IRS). Some data shows that the latter is more effective than the former especially when non-repellent insecticides are used[1]. When evaluated over longer periods, LLIN often have a superior effect when measured on malaria prevention[2] or entomological parameters[3]. Recent modelling showed that cost efficacy of the two treatments depends on user rate of LLIN, pyrethroid resistance and durability of IRS[4]. Spray campaigns are expensive, and the effect is often short. Since the insecticide cost is often just a smaller part and operational cost a major part, enhancing the durability of a spraying can reduce the need for repeated spraying and thus the overall cost allowing for a technically advanced and possibly more expensive product. The first product on the market of this type was a microencapsulated product with pirimiphos methyl developed by Syngenta[5]. However, the product was set at a high price and the sale was therefore limited which led the Gates Foundation (through the Innovative Vector Control Consortium, IVCC) to sponsor a part of the price to get it more used[5]. This support to countries and the company producing the product now includes a large group of countries in Africa. One reason for this support is that resistance to pyrethroids and DDT is on increase whereas the resistance to Organophosphorus products (OPs) like pirimiphos methyl is still low in most areas.

We aimed at developing a long-lasting IRS product based on an OP with low toxicity and a duration of at least 18 months. Since such test takes at least 18 months, we initially started with laboratory methods modifying the WHO test protocol to be able to detect promising formulations before a full year. The best candidates were then transferred to semi-field test in huts built in materials typically found for houses in Africa. We here report the development of the test methods from the laboratory phase to the semi-field tests and the experience drawn from that to help other product developing groups and laboratories to an easier process and everybody else to a better understanding on the interaction of the product evaluation processes and the product. We also report a success of our test program in developing an OP based IRS product with more than 18 months control of mosquitoes. The test program included a co-operation with Chinese research institutes that developed a similar product based on the carbamate bendiocarb. Bendiocarb is already widely used for wall spraying in Africa but has a disadvantage of a too short control period[6], hence the interest of making it long-lasting.

Methods

Non-pyrethroids insecticides recommended by WHO and the pseudo-pyrethroid etofenprox were used for the initial screening. Malathion technical grade (96%) and micro-encapsulated (20-30%, several formulations) were received from Cheminova (Denmark), Chlorpyrifos technical grade and micro-encapsulated was received from Makhteshim (Israel, now part of ChemChina), Chlorpyrifos-methyl and Phoxim from King Quenson, China, and Etofenprox from Mitsui Chemical Co (Japan), Bendiocarb micro-encapsulated from the joint Landcent Group and Shanghai Institute of Organic Chemistry, China. Malathion technical grade was formulated to an Emulsified Concentrate (EC) for initial methodological tests by the first author. Additives with coating effect were received from many companies: Silicone types from BASF and from BlueStar Silicones, acrylics and acrylic copolymers from Hexion (Spain) and detergents influencing wetting ability and droplet size from Croda, UK, and Lubrizol, France.

The study partner Capsulae developed the long-lasting microcapsules that were applied with chlorpyriphos and chlorpyrifos-methyl. The insecticide was microencapsulate using interfacial polymerization, whereby monomers are made to polymerize at the interface of two immiscible substances. In a first aqueous phase, a surfactant was dissolved in distilled water under stirring. In the oil phase, the insecticide and a monomer were dissolved in purified maize (corn) oil under stirring. A second aqueous phase was made with another monomer. The oil phase was added to the first aqueous phase under homogenisation (Ultra-Turrax) for 45 min to from an emulsion. Still under stirring, the second aqueous phase was added to start the polymerisation. Stirring was continued for 1 hour at room temperature. Capsule formation and size

were confirmed by microscopy.

As recommended by WHO[8], spray targets were inserted in a Potter Tower (Burkhard Scientific, UK) that by air pressure sucks the product from a small test tube and spray trough a steel nozzle with round orifice that provides a very uniform droplet size. The target was weighed before and after the applications to know the dosage obtained since a great part of the droplets end on the sides of spray tower. During these tests, it became evident that formulations that were just a bit viscous would not be sucked up in one constant flow, and the sucking device was replaced with a syringe to inject the test product into the small chamber with air pressure and the valve.

3 types of plates were used for the Potter tower tests: plywood, plywood painted with white acrylic paint to imitate a painted wall and plywood painted twice with white-wash chalk to imitate a concrete or white-washed house. The latter provides a high pH surface that is supposed to be destructive to many insecticides.

These plates were exposed to mosquitoes by fixing a WHO test cone on the plate for a fixed number of minutes. The WHO test protocol for test of IRS products ascribe 30 min exposure time. We tested 3, 5, 10, 20 and 30 min to see if a shorter exposure could give an early indication of what would succeed after 1 year with 30 min exposure. Duplicate plates were sprayed with the same product and the samples were sent to a bioassay laboratory in Thailand, Chian Mai University for the first years, and later to the Biolytrics laboratory in Hanoi ISO certified for using WHO test methods. The Thai laboratory used *An minimus* or *An cracens*, two replicates per plate each with 5 mosquitoes, thus 10 per recipe. The Vietnamese laboratory used *An dirus* or *An epiroticus* originating from the National Institute of Malaria and Entomology (NIMPE) in Hanoi, 6-7 mosquitoes per cone and total 50 per recipe. All strains were fully susceptible to insecticides.

Second phase were semi field tests in Burkina Faso in co-operation with Centre Muraz, Bobo Dioulasso. 8 test houses were built in the nearby village Soumousso, 3 with mud walls as most houses of the village, 2 with concrete walls as the few "modern" houses, one with red stone walls cut from local hard stones used in some houses, and one from wood. The control house had the 4 walls in the 4 different wall materials and was sprayed with water or water plus coatings only. When a test was finished for the concrete walls or the mud-wall house, it was re-plastered before next test. Wood and red stone houses were not used for repeated tests as explained below. As opposed to test huts used in the second phase of WHO IRS evaluation, these test huts only served to evaluate product durability and were not inhabited.

Each test house was 3 x 3.7 m², had one window with an open grid for ventilation and a door that was locked between tests to keep guests out (Fig 1). To prepare a test, a frame made of double folded tarpaulin with a hole of 75 x 75 cm (app 0.5 m²) was attached to the wall. A 15*20 cm Whatman paper was hanged approximate in the middle of the test field, and the field sprayed and the position of the paper was marked with painting, a different paint colour for each series of test. Each test field had an identity number painted on top of it (Fig 2).

Target dosage was 0.5 to 2 g ai/m², depending on the insecticide. 20-30 % concentrated products were diluted to obtain that 30 g sprayed would hold 0.25 to 1.0 g a.i. Most applications were carried out with a handheld sprayer IK 1.5 Professional equipped with a flat orifice nozzle (Goizper Group, Spain). The company helped adapting valves and pressure to ease the application of the products to the walls sprayed at 40 cm distance. The sprayer came with a pressure reduction valve, but there was no important pressure reduction during the spraying of the small test volumes. Using the pressure reduction valve, spraying starts with a second or more delay after activating the sprayer and this made it difficult to apply accurately. It was therefore dismounted. The sprayer was re-pressurized between each application till the security valve alarmed. The first author did all applications to get more constant results than would be obtained when different people spraying.

Three and for some tests 4 measures were taken to estimate the dosage applied: (1) the spray can was weighed before and after each application; (2) the Whatman paper hung in the spray area was weighed before and after each application; (3) the dosage insecticide on the paper was measured by GC-chemical analysis. Eventually (4), sticky yellow tape as used for

Figure 1. The concrete stone house, the red-stone house and the mud-house dimension 2.5 x 3 m in ground area.

Fig 2. Applying insecticide wit a flat fan, small volume sprayer on the wall field limited by a holled tarpaulin frame and with a Whatman paper attached at the middle of the spray field for collecting spray data.

paint protection was attached to the sprayed surface beside the Whatman paper, tapped and rubbed to obtain a maximal adherence of insecticide. The tape application was used in concrete houses to see if it provided additional information of the insecticide decay. Mudd walls were too rough for this measure.

Female *Anopheles gambiae* strain Kisumu (*kis*) were exposed to the sprayed surfaces under WHO test cones for 30 minutes. In the Standard WHO procedure, the mosquitoes are 2-5 days old, non-blood fed, but we also tested blood fed mosquitoes[8]. For each test field, 25 to 30 mosquitoes were introduced to 4 cones, and for each formulation, we had 1 to 5 replicates per house type. A product was considered effective if 80 % of the mosquitoes exposed for 30 min died within 24 hrs.

It was observed that in some cones, mosquitoes did not rest on the walls for 30 min and in some tests, were only exposed for 5-10 min, then sat on the cone and did not move back even when tapped. We decided to count mosquitoes on the cones after 10, 15, 20 and 30 min. The contact avoidance behaviour could then be analysed relative to formulations, wall surface and wall exposure to sun. Further, we tested blood fed and non-blood fed for this behaviour.

For most of the work, cones were fixed to the walls with sticky yellow tape as used when painting walls. At the end of the test, cones were fixed to mud walls by a paper ring with a hole the size of the cone and the ring was fixed to the wall using a staple gun with broad staples (Wall staple gun Fischer Dawex, staple type E12).

Bioassays were carried out with 1.5-2 months interval until exhaustion of the best candidates, typically around 12 to 18 months after spraying.

For the chemical analysis of the Whatman papers, a piece of 5 x 5 cm were cut out, cut into pieces of 1x1cm with acetone cleaned scissors, the pieces were mixed and transferred into a 100 ml cleaned bottle with 25 ml acetone, and placed in sonic bath at room temperature for 15 min. Paper was removed with stainless tweezer, the solution was transferred to an evaporation flask together with two rinses of 100 ml bottle and acetone was dried off in a vacuum rotator evaporator. 25 ml xylene was added, the bottle was placed in sonication bath for 15 min, one ml was extracted with a syringe with filter (0.45 um) and injected into a Gas chromatograph, type FID. Calibration curve was obtained from Sigma pure insecticides and the dosage was calculated back to mg/m² area of the paper.

Chemical analysis of the sticky tape was analysed the same way, but here the whole tape was used after measuring surface area.

This extraction method did not work for the improved formulation of bendiocarb and dosages could only be calculated from the known concentration in the micro-encapsulated concentrate and the amount sprayed on the whatman paper after dilution. The extraction method suggested by the producer did not provide reliable results.

Statistics

Anova general linear variance analysis, variance analysis for repeated measurements and correlation analysis were carried from Excel files imported to Statistix 10[9].

Ethics

Since the first author carried out all formulation work after micro encapsulation and all sprayings, no ethic committee acceptance weas demanded.

Results

Potter Tower Tests

Initial test with malathion EC at 1 g a.i./m² and exposure for 3, 5 and 10 min showed that 3 min would often give 0 mortality and 10 often 100 % mortality of newly sprayed samples, 5 minutes exposure time were chosen for initial screening. Second screening round applied malathion EC for 5 min exposure that showed that painted or raw wood surfaces gave much higher mortalities than white-washed wood surfaces (Table 1). Tests were stopped at 3 months as in the example in Table 1, or when mortality reached the 80 % criterium.

Etofenprox microencapsulated or as EC was tested at 0.3 and 0.6 g a. i./m². The EC formulation gave lower mortality on raw wood (50 % after 1 week) than on painted wood or white-washed wood (100 %), whereas microencapsulated gave the same mortality on the 3 surfaces (100%).

Further testing concentrated on micro-encapsulated insecticides tested on white-washed plates being the most challenging surface. Painted wood

was used for reference tests.

Microencapsulated Malathion, Phoxim and Chlorpyrifos-ethyl were tested on white-washed and raw ply-wood plates with or without the addition of various coating agents. It was found that whatever was applied on freshly white-washed plates, the insecticidal effect was short, and it was decided to paint plates at least 2 weeks in advance. A silicone additive combined with Phoxim or chlorpyrifos then provided a residual effect for up to 18 months with less than 30 min exposure. Table 2 shows the early phase of this development with high control till 12 months after 5 min mosquito exposure, then failed at 15 months.

Table 2 is an example of testing a microencapsulated insecticide, phoxim, on white-washed panels with a short exposure time of 5 min, but high dosages. There was a dosage effect of Phoxim, but not of the two additives except perhaps when combined. Discrimination between recipes started not before 1 year, the test dosages were too high despite the short exposure time. Chlorpyrifos provided results with the same efficacy as phoxim at similar dosages, whereas malathion provided slightly shorter residual effect.

However, the WHO-FAO registrant of Phoxim (Bayer, Germany) no longer supported the insecticide except for veterinarian use and was not able to provide new tox data needed for a renewal of the insecticide recommendation. Only Malathion and Chlorpyrifos were therefore transmitted to the test huts in Burkina, malathion to be tested at 2 g a.i/m² and chlorpyrifos 1 g a.I./m² since these dosages were efficient in the screening.

Hut Studies

Whatman paper 15*20 cm was fixed to the wall with a pin in the middle of the spray field (Fig 1). When removed after spraying, the area behind the paper was completely dry and uncoloured when whitish coatings were added to the spray formulation. The paper was weighed before and after spraying and this provided a quick estimate of the dosage applied calculated from the amount of spray solution applied to the Whatman paper. The paper was air-dried, wrapped in alu-foil and sent for chemical analysis. The dosage found in the chemical analysis was compared to the expected dosage from the amount of spray fluid picked up by the paper (Ratio 2 in table 3). Further, we compared dosages calculated from the sprayed volume to the dosage calculated from the amount on the paper (Ratio 1 in table 3). Table 3 is an extract including 8 applications (out of 64) to show the kind of data obtained from spraying in the test huts, but the summary line includes all 16 applications made this way. The insecticides were microencapsulated malathion and chlorpyrifos, targeted dosages were 2 g malathion/ m² and 1 g chlorpyrifos/m², combined with various silicone coatings or without and tested in 4 types of huts.

The malathion average dosage obtained was 1.94 +/-0.25 g malathion/m² measured in the paper extract thus close to the target 2.0 g/m². The ratio between dosage calculated from volume sprayed to calculated from what hit the Whatman paper showed a loss of 28 % in average, ratio 1. These applications were made with a first handheld sprayer with round orifice nozzles and we therefore changed to the IK sprayer with flat nozzle for the rest of the study. That reduced the loss from applications to 15 %. Further, less insecticide was found on the paper than calculate from the amount hitting the paper (Ratio 2) showing a problem of uneven droplets in dosage or sedimentation in the bottle.

The full test included 64 datasets testing dosages of acetic acid and coatings and malathion and chlorpyrifos. Impact of wall type and insecticide was first analysed in a variance analysis[8]. The 5 min exposure mortality data obtained 2 days after spraying (0 months in Table 3) had wall type as a significant factor ($P<0.001$), but not of insecticide or formulation. Data for the 5 min exposure mortality after 3 months had significant impact of wall type ($P<0.0001$) and of insecticide formulation ($P<0.001$), 6 month data could not be analysed this way because of missing data (not enough mosquitoes), 9 month data of mosquitoes exposed under cones for 30 min again had significant impact of wall type and insecticide ($P<0.0001$), whereas at 12 months, only wall type was significant ($P<0.05$). 24 months data were only taken for the wood house, but the effect was generally very low except for a few chlorpyrifos applications (max mortality 80 %).

Least Significant difference (Tukey-test, significance level set to 5 %) tests were carried out for the variance analysis of 3, 9 and 12 months and showed that the wood walls always provided the highest

Table 1. M24hr is mortality after 24 hr, malathion EC was applied at 1 g a.i./m². Painted wood provides 100 % mortality, raw wood less and white-washed wood with high pH, low mortality.

Time since spraying	1 week	1 month	3 months
An species	*Cracens*	*cracens*	*minimus*
Surface	M24 hr	M24 hr	M24 hr
Painted Wood	100%	100%	100%
Raw wood	100%	80%	60%
White-washed wood	5%	20%	5 %

Table 2. Testing microencapsulated Phoxim sprayed on whitewashed panels adding various coating types and/or dispersing agents.

Coating type	Dosage g/m²	Phoxim g/m²		Mortality 24 hr % after 5 min exposure and X months			
			1	3	6	12	15
None	0	2	100	100	100	75	10
None	0	4,8	100	100	100	100	45
Acrylic	2	2,2	100	100	100	50	5
Acrylic	2	4,9	100	100	100	100	45
Acrylic	6	5,4	100	100	100	100	15
Acrylic	8	5,3	100	100	100	100	20
Silicone	2	2	100	100	100	85	20
Silicone	2	4,9	100	100	100	100	0
Silicone	6	4,9	100	100	100	100	20
Silicone	8	5,1	100	100	100	100	30
Acrylic+Silicone	3 + 3	4,5	100	100	100	100	60
Acrylic+Silicone+ Dispensing agent	3 + 3	5	100	100	100	100	70

mortality and mud the lowest, with concrete and red stone in between, but only significant higher than mud walls after 12 months.

The mortality data for 5 min exposure after 3 month and the mortality after 30 min exposure 9 and 12 months provided the same results for these two criteria. Correlation analysis between mortality at 3 months and 9 month and 3 months and 12 months showed high significance ($P<0.0001$), though explained variation r^2 were low, 0.36 and 0.35 for the two correlation, respectively. Thus, the 5 min exposure test at 3 months can serve as a rapid test for what will happen up till 12 months in a standard 30 min exposure test, but the predictive value is not exact.

Impact of additives were analysed per insecticide and wall type, but no significant impact was found on mortalities at 3, 9 or 12 months.

Some of the best formulations from the Potter tower screening test are compared to results from these hut tests (Table 4). The table shows results of Potter tower sprayed malathion plywood gave 100 % control up to 2 years after 15 min exposure, but after 9 months on concrete walls only 30 % control after 30 min exposure. Chlorpyrifos on white-washed Potter Tower sprayed samples tested at 5 min exposure provided for 2 years 100 % control, but the same formulation gave between 10 and 90 % control after 14 months in the huts, thus a shorter residual effect and with much variation. In general, the laboratory studies vastly overestimated the residual effect in the huts and best recipes were often not the same.

To be able to follow insecticide decay over time, bioassays had to use a constant exposure time and we chose to use the 30 min as in WHO standard test for the hut tests[8]. Further, we increased the number of repeats per formulation and dropped the wood house and later the red stone house, because they provided the least critically information: wood applications lasted for ever and red stone data were not different from concrete wall data. The table below is an extract of trials where the insecticide was micro encapsulated Chlorpyrifos-methyl and the coating additive was either mixed into the sprayed products or partly applied an hour before to obtain that the wall was coated before the spray was applied. The coating additives added as pre-treatments represented a silicone type and a polyurethane type, only the silicone type was mixed into the sprayed product.

As indicated in table 5, analysis of all data set (48 applications followed in 14 months) applying the coating additive before and with the spray did not improve the residual effect compared to having all in the sprayed products, and the residual effect in general was high even after 14 months.

General Anova Variance analysis of all datasets showed that for 3 and 6 months, wall type was a significant variable with mud wall providing the lowest mortalities. However, the significance disappeared after 9 months and could not be analysed after 14 months where one house was taken out by mistake. The Anova analysis showed that pre-treatment with the coating or coating integrated made no difference. Using the varians-analysis as a guide, wall type was classified 1, 2 and 3 and the mortality data were analysed in regression analysis. These showed as above that wall type impacted mortality, whereas insecticide dosage only significantly impacted mortality after 9 months ($P<0.05$) and otherwise just showed a tendency ($P=0.15$). Dosage of coating was not a significant parameter. Overall regression was low, $r^2=0.41$ for 3 months declining to 0.20 for the rest of data.

The products tested above were based on a 27 % microencapsulated concentrate that was too viscous for a commercial product. The micro-encapsulation laboratory therefore provided a new concentrate of 33 % that was sprayable when formulated at 27 %. This product was tested on mud walls and concrete walls in parallel with a bendiocarb micro encapsulation formulation from a Chinese company.

The microencapsulated chlorpyrifos methyl still gave close to 100 % control after 18 month and above 80 % after 26 months in all formulations on mud walls. Table 6 shows mean values for 4 applications of 3 different ways of microencapsulating chlorpyrifos. MC 0 were made without coating, MC + and ++ with medium and high level of coating. The 26 months data showed that MC + was significantly better than MC 0. The micro-encapsulated bendiocarb declined after 6 months, then apparently regaining after 12 months.

To see impact of pH of the formulation on durability of chlorpyrifos methyl on 3 mud-walls and 2 concrete walls per recipe, the recipes were pH adjusted

Table 3. Silic is short for silicone additive, Spray is amount sprayed, Wet is the amount hitting the Whatman paper, and Chem is the dosage on the Whatman paper as found in chemical analysis, all converted to g a.i./m². The average data presented are calculated from 16 applications. The two ratios indicate spray losses. The bioassay results of short time exposure in the start is compared to the results with 30 min exposure as carried out later.

Mortality 24 hr after X months/exposure Y min													
Recip	Wall	Additiv	Spray (g)	Wet paper	Chem Analys	Ratio 1	Ratio 2	*0/5m*	*3/5m*	6/30 m	9/30 m	12/30 m	24/30m
508.1	cement	0	3.23	2.63	2.13			*50*	*5*	46	15	14	
508.2	red stone	0	3.35	2.90	1.99	1.26	1.36	*75*	*0*	Nd	11	16	
508.3	Mud	0	3.23	2.44	1.75			*65*	*5*	Nd	19	13	
508.4	wood	0	3.12	2.26	1.63			*100*	*10*	100	88	85	20
509.1	cement	Silic	3.35	2.90	2.34			*45*	*0*	55	38	27	
509.2	red stone	Silic	3.23	2.54	1.98	1.24	1.25	*80*	*5*	Nd	25	30	
509.3	mud	Silic	3.46	2.90	2.38		0	*80*	*0*	Nd	33	13	
509.4	wood	Silic	3.46	2.54	1.96		0	*100*	*25*	100	68	50	40
Average of 16 applications			3.13	2.64	1.94	1.28	1.26						

Table 4. MC+ is microencapsulated, Lab WhiteW is plywood samples white-washed, Time is exposure time for mosquitoes and result are mortality in percentage at months (M) after application.

Table 4 Insecticide	a.i./m²	Formulation	Surface	Time (min)	Results (Months, % Mort)
Malathion	2	MC+	Lab WhiteW	15	24 M 100 %
Malathion	2	MC+	Field Concrete	30	9 M 30 %
Chlorpyrifos	1	MC+	Lab WhiteW	5	24 M 100 %
Chlorpyrifos	1	MC+	Field Concrete	30	14 M 10-90 %
Chlorpyrifos	1	MC+	Field Mud	30	14 M 10-90 %

Table 5. Analysis of all data set (48 applications followed in 14 months) applying the coating additive before and with the spray did not improve the residual effect compared to having all in the sprayed products, and the residual effect in general was high even after 14 months.

Nr	Wall	Dosage ai/m²	Coating	Dosage g/ m²	Mort 1 M	Mort 3 M	Mort 6 M	Mort 9 M	Mort 14 M
579,1	Concrete	0,4	Silicone	4,37	100	100	96	80	100
579,2	RedStone	0,468	Silicone	4,72	100	100	79	89	
579,3	Mud1	0,351	Silicone	3,67	100	48	100	100	100
579,4	Mud2	0,637	Silicone	4,72	100	55	88	100	91
583,1	Concrete	0,549	Silicone	8,82	100	100	83	90	91
583,2	RedStone	0,658	Silicone	8,82	100	100	100	100	
583,3	Mud1	0,421	Silicone	6,32	100	43	90	85	81
583,4	Mud2	0,421	Silicone	6,47	100	70	100	100	100
585,1	Concrete	0,433	Silicone	2,91+3,41	100	100	88	55	100
585,2	RedStone	0,462	Silicone	3,30+3,41	100	100	86	85	
585,3	Mud1	0,484	Silicone	2,76+3,41	100	61	100	86	87
585,4	Mud2	0,464	Silicone	1,38+3,41	100	67	87	100	100
587,1	Concrete	0,415	Silicone+Polyurethan	2,71+8,79	100	100	88	90,48	96
587,2	RedStone	0,417	Silicone+Polyurethan	3,05+8,79	100	100	88	84,21	
587,3	Mud1	0,492	Silicone+Polyurethan	3,10+8,79	100	52	95	100	67
587,4	Mud2	0,656	Silicone+Polyurethan	3,30+8,79	100	100	100	100	100

with citric acid and 2 g coating/m² (CPM 19). The bendiocarb recipe (Bendio4 below) was adjusted to pH 5.0 and presented a new micro-encapsulation method. The formulations made without the coating were also tested after 638 days and provided around 35 % mortality. These bioassays were carried out with the usual test strain *An gambiae kis* except for the 9 months test where we used the multi-resistant lab strain VKPER (Table 7). Clearly, the impact on VKPER after 9 months was lower than that on the fully susceptible strain after 6 and 12 months. Further improvements of the bendiocarb encapsulation were tested on mud and concrete walls with and without a coating additive. Twenty four months after application of this product that was pH corrected by the producer, the formulation without the coating additive performed slightly better (75 % mortality) than the one with the coating additive (60 % mortality, P<0.05) and better on mud walls than on concrete (74 % vs 62 %, p<0.05). The result on the mud wall was 80 % control. This was confirmed in a second trial that ended after 531 days. The target dosage was 1 g bendiocarb/m² and the calculated dosages were between 0.9 and 1.1 g bendiocarb/m², but could not be confirmed by chemical analysis as explained under methods.

Finally, another type of coating that was less expensive was added to the same SC, Table 8. The table show dosage measurements from paper attached to the spray fields, and mortalities as registered after 30 min exposure 630 days after application; before that, mortality was 100 % on all mud walls but lower on concrete.

with General linear varians analysis followed by Tukey least significant tests (P set to 5 %) showed that dosage of the two products and on the two surfaces (5 repeats) were not different, but mortality was higher for the product without coating than with this new coating, and it was higher on mud. Numbers followed by same letter are not different. Linear regression analysis showed that within the groups Mud and Concrete, there was no correlation between dosage found on paper and mortality.

Resting Time on Walls

It was observed that in some cones, mosquitoes did not rest on the walls for long time, and after 10 min, most would stay on the cones, even these were tapped. Real contact time was thus not 30 min but whatever the mosquitoes accepted. To see if this was related to formulation, we counted number of mosquitoes resting on the cones for every 5 min starting after the first 10 min, Table 9, that shows two series of tests are from two mud houses and one concrete house

A Pearson correlation analysis of the resting time data showed that mean number and max number in the same spray plot correlated well, but neither mean number nor max number correlated per recipe between houses. Specifically, the repeats for the two mud houses did not correlate (linear regression P=0.85). Finally, the number of mosquitoes on the cones were not correlated to mortality for all walls nor specifically for concrete walls, where mortality was below 100 %. At this range of mortality, there were no significant correlation between cone resting mosquitoes and mortality, for the concrete wall only, P=0.17. Resting time thus did not depend on formulation.

Recipes were sprayed on the test plots in a randomized way on the 4 walls of the house. At the next round, the number sitting on cones were analysed according to wall. That showed that on one wall in the mud house, 5 different recipes had in average 34 % of mosquitoes sitting on the cones after 15 min and 100 % after 25 min (of those not knocked down), whereas on the other walls only 1 to 4 % were on the cones after 15 min and 1 to 10 % after 25 min. In the wood house 50-100 % of mosquitoes on one wall were on the cones after 15 min, compared to 5 to 20 % on two other walls. Finally, in the control house, after 15 min on the wood side, all mosquitoes were on the cone side, compared to 0 for the concrete and mud walls.

These results showed that sitting on cones were not due to formulation, but to walls. Mosquitoes sat less on walls that were sun exposed and especially so if the wall was out of wood, probably because wood wall heated more than the other walls. We therefore repeated some tests made early afternoon with test early morning the day after, same positions, and the sitting on cone problem disappeared. From that on, cone tests were stopped before midday on sunny days.

Impact of Blood Feeding

Because of the problem with cone resting and possible implication for mortality data, we repeated a house test with blood fed mosquitoes. Blood fed

Table 6.Three formulations with microencapsulated Chlorpyrifos methyl with no coating (MC 0), medium level coating (MC+) and high-level coating (MC++) and three different microencapsulated bendiocarb formulations were followed by cone assays, 24 hr mortality over 26 months.

Recipe	g ai/m²	0 Mo	1.5Mo	3 Mo	4.5Mo	6 Mo	12 Mo	18 Mo	26 Mo
MC 0	1.12	100	100	99	100	98	100	99	65
MC +	1.33	100	100	100	99	99	99	99	90
MC++	1.17	100	100	100	99	98	100	90	74
Bendio-1	1.12	100	100	69	81	63	93	ND	ND
Bendio-2	1.29	100	100	48	77	64	82	ND	ND
Bendio-3	1.37	100	100	69	87	65	95	ND	ND

Table 7. Impact of pH in formulations applied on mud and concrete walls.

Recipe	Wall	0 Mo	4.5 Mo	6 Mo	12 Mo	9 Mo (VKPER)
CPM 19 pH6	Mud	100	100	100	100	47
	Concrete	100	100	100	100	
CPM 19 pH5	Mud	100	100	100	100	48
	Concrete	100	100	100	100	
CPM 19 pH4.5	Mud	100	100	100	100	25
	Concrete	100	100	100	97	
Bendio4 w coat	Mud	100	98	100	95	6
Bendio4 w coat	Concrete	100	94	100	90	
Bendio4 no coat	Mud	100	98	100	100	8
Bendio4 no coat	Concrete	100	100	100	100	

Table 8. Effect of a cheaper wall coating additive on mud walls, mean dosage and mortalities 24 Hr. The brackets indicate significant differences, numbers with the same letter are not different, Tukey Test 5% significance.

Product type	Dosage g a.i./m²	Mort 24 hr
No coating	1.09[(a)]	61 %[(a)]
Coating	0.89[(a)]	49 %[(b)]
Surface Type		
Mud	1.02[(a)]	84[(a)]
Concrete	0.93[(a)]	25[(b)]

Table 9. The mean number of mosquitoes resting on the cones over the 4 counts (10, 15, 20 and 25 min) out of 25 mosquitoes in the 4 cones per spray field, the max number and the 24 hr mortality.

Nr	Mud 1			Mud 2			Concrete		
	Mean	Max	Mort	Mean	Max	Mort	Mean	Max	Mort
600	0	0	100	2,5	10	100	31	33	95
601	6	10	100	5	15	100	26	30	95
602	8	14	100	5	10	100	16	27	91
603	8	10	100				36	38	95
604	21	25	100	0	0	100	24	33	94
605	10	15	100	18	25	100	11	21	100
606	18	22	100	17	32	100	16	17	100

mosquitoes did not move but stayed on walls that non-blood fed would only sit on shortly and the mortality was higher.

Fixing Cone on Walls

We initially fixed the cones on the walls with painter's tape that is easy to remove again from the cones. On smooth walls of wood and concrete, the cones are easily fixed tightly to the walls this way. However, mud walls have a very irregular surface and the sticky tape attach badly because it may simple remove an upper dusty layer and the cone may fall off or open partly. The biggest problem was that the cones could not be fixed closed to the irregular wall surface so fainted mosquitoes could drop down on the sticky tape. One to 3 mosquitoes were often stuck on the tape and could not be safely recovered. Trying to remove them may tear of legs and wings and thus lead to increased mortality. We had to exclude them, but this probably leads to underestimated mortality since they were those knocked down early.

Therefore, we produced carton paper frames with a hole the size of the cone and these were fixed to the wall with a staple gun with broad staples. Narrow staples shot through the paper. Further, this was faster than using sticky tape. We observed no data problems with this method.

Further Chemical Analysis

We tried to follow the decay of insecticide by using sticking paper that was pressed by thumb on the concrete walls and mud walls. Two pieces of sticky tape was attached to the wall close to the Whatman paper, one just above it and one just beside it. The position was marked with a speed marker to avoid sampling from the same area again later.

The chemical analysis showed that the sticky paper only picks up around 10 % of what was found on the Whatman paper beside. Because of the high variation in these data already from start and the low fraction picked up, the method was given up since any decay would disappear in these variations.

Discussion

This article has two aspects. One is how to test new IRS formulations starting with the WHO prescribed Potter tower[8] and then comparing the data obtained in test houses. Since these were different, we developed methods for screening in uninhabited test houses. The second aspect is the development of two long-lasting IRS formulations.

Formulations of insecticides were initially screened using the Potter tower as recommended by WHO. To gain time, we exposed mosquitoes for short time instead of standard 30 min anticipating that long lasting formulations would decay slower and be identified after months instead of after years. The initial screening showed that this worked since discrimination of surfaces (raw wood versus white-washed wood with high pH) and of formulation (micro-encapsulated versus EC) came out as known from the litterature[10, 11].

However, when these best formulations of two OPs were transmitted to hut tests, the durability was much shorter (Table 4). Therefore, a new screening was set up in 4 types of huts with walls of wood, stones, concrete, or mud. These tests also started with short time exposure and compared the results to those obtained later with 30 min exposure. Correlation studies showed that there was a significant correlation of the mortality results obtained with short exposure time in the start of the evaluation to long exposure time later (Table 3). However, the correlation coefficient was low and it was therefore decided to drop this short cut.

Spray dosages were estimated on the spot by weighing the sprayed paper and by weighing the spray can before and after the spray. These comparison showed that the first hand held sprayer with round valve was not suitable and we replaced it with the IK sprayer that was used for the rest of the tests and is a mini version of a 10 litre sprayer of the same company. Further on, formulations were discarded if these numbers were too different since that indicated that many droplets did not reach the wall.

Chemical analysis of the Whatman paper in the middle of the spray field (Fig 2) and of painters tape used to pick up insecticide from the walls showed that the latter method was not providing useful data since only 10 % of the insecticide was picked up and with big variations. The adhesive tape method has been used in field studies of sprays to show spray decay over time[6,12], but with this deviance in dosage, this is not judged to be informative. However, it is possible that Thawer et al al did not use the adhesive tapes as explained in their text and figures but only scraped off the wall surfaces, the

alternative method. The study of Russell et al[12] confirms our results.

Chemical analysis of the paper was also used to compare to the dosage obtained from weighing the paper before and after spraying. Except for the tests with the first handheld sprayer (Table 3), the results were nearly the same. Weighing the paper before and after spraying can thus serve as a good field guide of spray dosage obtained.

The tests in the 4 types of test houses with mud walls, concrete walls, red stone walls or wood walls showed that insecticides on wood walls remained active for much longer time than on the other walls and thus were dropped for screening. Further, the red stone walls and the concrete house walls gave similar results, so the red stone house was plastered with concrete. Concrete houses and mud houses were re-plastered between test to avoid any problems with insecticide residues.

To obtain many spray fields needed for screening, a moveable frame was used to limit test fields to 0.5 m² (Fig 1). In this way, we obtained 25 test field per house. During the initial screening, each recipe was repeated once in each of 4 test houses. Later in the product development, this was increased to 4 and finally 5 repeats in two types of test houses, concrete and mud, to provide significant comparisons.

Since we followed cones closely, we discovered problems with cone assays on walls we have not seen reported elsewhere. The bioassay data showed a lot of variation. We found that the time the mosquitoes spent on the walls varied, not with formulation but with geographic orientations of walls. Walls that were sun-exposed had mosquitoes refusing to sit on the walls and they instead sat on the cones, even cones are made of PVC that they should not like to sit on. This problem was solved by stopping bioassay before midday, but it is likely that most programs with field evaluations of IRS spray do not observe their cones closely and thus have a non-recognized source of error.

We tried to replace non-blood fed female mosquitoes with blood fed the same morning. These females would sit on all walls for 30 min on walls sun-exposed or not opposite to the non-blood fed, and they showed higher mortality than non-blood fed. The WHO standard protocol[8]use 2-5 days old, non-blood fed mosquitoes. Recent data[13] from *Culex pipiens* showed that some strains had increased insecticide sensitivity after blood meals while others had decreased sensitivity of an order 10 to 50 in topical application tests. Test of single blood meal to *Anopheles arabiensis* showed significant increased resistance after 3 days for pyrethroids and DDT, but less for malathion[14]. Therefore, the results using blood fed or not can make a difference not just because of their resting behaviour. For *Anopheles spp,* IRS target blood fed mosquitoes, not non-blood fed that fly into houses to bite and after biting retire to the walls to digest. It would therefore be more logic to test with blood fed mosquitoes than using non-blood fed as is now the WHO standard protocol. Not to open a debate on the validity of our main findings, we did however continue to use non-blood fed females.

Another problem with the cone bioassay is the way it is fixed to the wall. Mud walls have a very irregular surface, so when fixed with tape, fainting mosquitoes easily fall on sticky tape. Trying to move these to cups for 24 hrs evaluation of mortality, legs or wings are easily teared off and create an overestimated mortality if included and a potentially underestimated mortality if excluded. We therefore made a hard paper frame with a hole that fitted the cone and stapled this to the wall with broad staples. That solved the problem.

The second aspect of this work was the development and testing of long-lasting IRS. Four OPs, one carbamate and one (pseudo)pyrethroid were tested as EC, WP or micro-encapsulated. Micro-encapsulated products performed better on white-washed plates and on mud-walls than those not micro encapsulated except for the pseudo-pyrethroid etofenprox. Etofenprox is chemically not an ester as the pyrethroids, the OPs and the carbamates, so this showed that hydrolysis on sprayed surfaces can be an important decay mechanism and does not impact non-esters like etofenprox.

We therefore tested various coating materials from the painting industry either as pre-treatments or intermixed in the formulation to protect against such destructions, since the insecticide must leave the micro-capsules to be active. These tests showed that there was no enhancive effect of adding the coating before the insecticide spray, which of course is an operational advantage. Further, that the added effect was higher when the treated surface was fresh. This aspect was later confirmed by Sumitomo (Lucas, pers. Communication) that had measured pH on concrete surfaces and

showed that after 2-3 weeks, the surface pH had declined from above 10 to around 7 (neutral), which neutralize the destructive effect (hydrolysis) of alkalic surfaces on many insecticides.

The tests in mud wall houses showed that we could provide a coating mixed product that after 26 months gave 90 % mortality of fully susceptible *Anopheles gambiae* females with the OP insecticide Chlorpyrifos methyl and 12 months with the carbamate bendiocarb. However, improved micro-encapsulation of the carbamate resulted in a product with residual effect up to 24 months but without any coating. Simple formulations of Bendiocarb are widely used for wall sprayings in Africa but the residual effect is normally below 3 months[6,15]. Increasing the residual life of this insecticide is thus very interesting.

An gambiae kis is the standard strain used in most of Africa for bioassays of products, but it is also a very old laboratory strain and the predictive effect against wild flying mosquitoes is unknown, it only measures the presence of the insecticide by its mortality. A product for two seasons must last at least 18 months and the number of spray applications in many countries with such a product can then be reduce to every second year which will be a major cost saving. This also applies to e.g. the control of triatomine bugs. Further, it has recently been shown that IRS can be used for *Aedes* control thus possible against dengue[16] where the mostly used aerial fogging has no residual effect.

In the final tests with 5 repeats per formulation (Table 8) with the variations caused by the manual spraying at +/_ 35%, we found no dosage effect on mortality when dosage was measured by extracting the insecticide from the Whatman paper or simply be weighing the Whatman paper before and after the spraying. That confirmed observations from previous tests reported in Table 5. The reason is probably the rough surface of concrete and especially mud walls. These are full of folds and fissures and the real surface of the 0.5 m² field is much larger, especially for mud walls, but very variable. This dilution effect may be one reason for a lack of significant dosage effect at this scale. It may explain why mud walls are generally accepted as being the most challenging to treat, simply because the real dosage per unit area is much smaller than the planned.

The first mosquito control product recommended by WHO and based on micro-encapsulation of an OP (Pyrimiphos-methyl) provided longer lasting effect than the same product delivered as a SE (suspension emulsion)[5]. We tested various micro-encapsulated products currently used in the agricultural industry and said to be long lasting, but the effect was too short. This is probably because long-lasting in agriculture is a few weeks since after that, plants has grown new leaves and must be re-sprayed anyhow. By developing special long-lasting capsules that let the insecticide migrate over many months, we obtained a consistent effect for nearly 2 years. That also meant that the capsule walls became a bigger part of the formulation and defined a limit to how concentrated the product could be made. On the other hand, in our trials this product provided much longer residual effect on mud walls than reported with any other insecticides. It can be expected to last for two seasons in areas without high OP resistance, currently most of Africa as seen on the Global map of insecticide resistance with the filter Organophosphorus, published by WHO[17], but this of course has to be confirmed. The same map with carbamates as filter shows more insecticide resistance areas, but Bendiocarb can still be applied in large parts of Africa with success, e.g. bendiocarb IRS was applied with significant impact of biting rates in Benin in areas with high pyrethroid resistance[18].

Beside a longer durability of the residual spray, micro-encapsulation improves storage ability and tox profile. This is one of the reasons the technology is widely used in agriculture[19]. Along with this, the producer of the bendiocarb microencapsulated product had their product tested at the GLP laboratory …. The study showed that the bendiocarb product did not have any acute toxicological effects.

The future of this project depends on the willingness of chemical companies to provide WHO with the needed tox data. After our study finished, EU decided not to re-register Chlorpyrifos and chlorpyrifos-methyl whereas EPA still sustain these. It is possible that WHO will accept Chlorpyrifos-methyl for this application, though Chlorpyrifos probably will be abandoned as indicated to us during the product development.

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