

**Insecticide Resistance:
Fourth MIM Pan-African Malaria Conference
Yaoundé, Cameroon
November 16, 2005**

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LIZETTE KOEKEMOER: And our first speaker today is Professor Janet Hemingway, and it's my honor to introduce her to you as an invited speaker. I'm sure most of you have heard about her. Anybody that works in resistance or in the field of vector control is familiar with the research that Professor Hemingway has done. And she's here today to talk a bit about the newly funded grants funding that they received. Janet.

JANET HEMINGWAY: I'm going to hold this. It's not doing anything at all at the moment, I'm afraid. But hopefully, if it turns on I'll deafen you all, and if it doesn't, I will shout. So hopefully my voice will hold out for the next 20 minutes or so.

But as Lizette said, what I wanted to do was to talk to people about the newly funded program that we have. My apologies for not putting a title in the program. When I was invited to come and speak, we weren't sure exactly when the announcement would come through, so I couldn't actually put a title or an abstract in the sort of system. But what has been funded ... Is anything happening? It now sounds as if something's going on. What has been funded is something called the Integrated Vector Control Consortium. We, over the last few years, have got used to calling it the IVCC, and hopefully

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everybody else will think of it in that way fairly shortly, in the same way people have got used to MMB and the vaccine initiatives. Could I have the next slide, please?

What I wanted to do is just give you a bit of background about why we have the program funded to start with, and then give people some idea of what the program is intended to do. At its very simplest form, what we're doing is developing better control tools for malaria and dengue vector control and learn better ways of actually implementing those tools. So there are two sides of the consortium that we're looking at. And part of the reason we're doing that is clearly that although we've all said we're trying to improve malaria control and trying to bring down the incidence of transmission, clearly in many areas we're not managing to achieve that. And part of that is clearly down to the poor vector control tools that we have and the poor way that they're being implemented in many places. Next slide, please.

We've got various issues to contend with, not least resistance to pesticides. So what you've got here is just the pyrethroid resistance that was occurring several years ago; the red area will sort of show you where we've had problems. In fact, it is worse than that. We have more resistance problems than that coming through, and clearly we have to make sure that we have tools and technology to be able to overcome that

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problem in exactly the same way as the parasitologists have had to do that with the drug resistance that's coming through in the parasites. Next slide, please.

So if we look in particular areas—this is, for example, India—we've got a lot of DDT resistance—those are the red dots that you see there—that came from the earlier wave of DDT use. And in many areas in India now we have pyrethroid resistance coming over and above that. That's some of the green dots that are coming in through there. But still in those areas, both DDT and pyrethroids are being used for many years. And it's becoming clearer, I think, to the people in India that we're getting control failure because of that resistance, certainly in some states. Next slide, please.

Similarly with Africa. If you look there at the presence and absence of the kdr mutation, you've got the red dots indicating where there is kdr, the green dots where we know that there isn't kdr. There are quite a number of areas where we have no dots at all, so we have no idea, really, what the incidence of resistance is in those areas. And the blue dot that we've got down in the southern end of Africa, in Mozambique, indicates where we have no kdr, but we do have a high level of pyrethroid resistance due to a different mechanism there. Next slide, please.

A few years ago, Pierre Guillet and Morteza Zaim, both

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now working for the WHO, toured around the different major manufacturers—this was in 2001—to try and find out what kind of new insecticides were likely to be coming through the pipeline from industry to replace pyrethroids, on either bednets or for indoor residual spraying. And the answer to that was basically very little. If we didn't help to stimulate that pipeline, and actually work with industry to try and improve not only the ways in which new active ingredients were coming through the system but the rate at which better formulations were sort of coming through. We know, for example, for mainstream vector control, both in indoor residual spraying and for insecticide-treated nets that pyrethroids really are the state of the art, and pyrethroids were built in more than 25 years ago. So we've had several decades now without any new classes of insecticide coming along. We're still working with the indoor residual sprays in many cases with 20-year-old formulations. And we're certainly using 30-year-old technology with indoor residual spray systems. So I think there is plenty of room for innovation there, in terms of improving what we do. Next slide, please.

There's also the side of the equation that says not only do we need better insecticides but can we actually use those resources better. And I think this is a great example for Mexico on how you clearly can use resources better; use

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less resources but get better control out of it. So what you're seeing here, and I hope people towards the back can see, the yellow bars we've got there, where it says eradication at the bottom, were in the 19 sort of 50s through into the 1960s and early 70s, when the malaria eradication campaign was going. And that was largely blanket coverage of indoor residual spraying with DDT. And the red line that you've got superimposed on top of the solid bars is the malaria transmission data. You can see when they were getting very high coverage, all [inaudible] within the sort of spray program and the DDT. They were managing to damp down the number of malaria cases; that's on this side of the axis, and it's starting from 20,000 cases and going up to 180,000 cases at the end. But you can see as the yellow bar starts to decline, and people not only in Mexico but worldwide decided that eradication had failed and the spray coverage reduced, that there was a massive upsurge in the amount of malaria transmission.

Over the last sort of 15 years or so now, we've been working in Mexico with the control program. Toward the latter end of that, we put a resistance management program in place. That's where the sort of green chunk is at the bottom there. And we've also, working very closely with the malaria control people, said, "How do we best target the indoor residual

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spraying that we're going to do?" So rather than trying to spray every house, we've sprayed the houses only where we believe the greatest level of transmission is going to occur. And you can see that by actually spraying a great deal fewer houses than we did in the eradication era, we've brought the malaria transmission right down. And even now going to doing very, very few houses within that sort of set, we're keeping that transmission down. We haven't had a massive resurgence of malaria. And I think that's a great example of how you can both control the resistance that you've got there and use limited resources well. Next slide, please.

So what are we actually going to do in terms of taking this thing through? Well, we're going to work with the chemical companies. So this isn't academia going to make new insecticides on its own and then set itself up as a chemical producer and marketer. We are not going to do that. Clearly, there is a whole big machinery out there from the chemical companies that does that very well. And the idea is very much that we set up a public/private partnership with industry on the new insecticide side. This is the sort of scheme as it goes through in terms of industry, from early discovery phase, through optimization, into field trials, the project phase and through into launch. We're not going to go right back into the early discovery phase, but we're certainly going to go into

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that optimization and investigation phase and all the way through from there.

I should say that in that particular area, as yet we have no projects that have been pre-selected for funding. We really are going to be going out there, talking to industry, talking with all the academic partners, and then we're going to set up a system that allows us to evaluate all the possible projects that we could put together, and pick the best of those to put into our pipeline. Next slide, please.

At the moment, if you look at what's happening with the vector control for dengue and for malaria, there are really three ways that people are doing it. For dengue, there's a lot of space spraying been going on. We're not, I don't think, going to be working primarily in that area. We are going to concentrate very much on the powey [misspelled?] domestic area and look at saying, "How good can we get both dengue and malaria control," just concentrating on that area. So most of our efforts will be in these latitude categories: the residual spray area and the insecticide treated materials, and that does include nets as well as curtains and other sort of sets that we may want to look at. Next slide, please.

Just again to give you some idea of the relative size of those three markets, the blue one on the bottom is the indoor residual sort of market, and you've got the 2003 actual

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figures here in terms of millions of euros. And while we're predicting by 2009 the markets will be, the green bars are the space sprays and the red, the insecticide-treated materials. And you can see from that that we believe, and this is worked out with industry, that the indoor residual spray market and the space spray market will stay pretty much as they are. And we're going to see growth in the insecticide-treated materials sort of side.

Obviously, it's a science that is not sort of completely exact in as much as we're dealing with attendables [misspelled?] in some of these sort of sets, and we do know that things can shift a little bit. But I think that by concentrating on the major areas in both sort of sets, we're not pushing either the treated nets or the indoor residual sprays. We're saying both have a very real role to play in control.

Now why do we need to work with industry? Why doesn't industry do this for itself? Well, it's fairly obvious that industry has gone through quite a contraction in the last few years. I've yet to tell you how many years it is I've started in this business, but it's a few decades ago. And a few decades ago, I probably had about 40 different companies that we could work with. But the number of companies that we can now work with is much, much more limited. And part of the

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reason for that is that there's been merges going on within the industry. So just to give you one example, Bioenvironmental Sciences, as it is today, is made up of all the companies you see below there, and you have merged together. What that also means is that the insecticides that those companies that made up Bio now have been reduced, in as much as one company is not going to support multiple product that compete directly with each other. And so we've actually had a reduction in the numbers of insecticides within each class that are available for vector control.

There are also only a relatively small number of companies in the market. These are the major insecticide companies that are out there at the moment. There are obviously many more smaller ones. And you can see where the blue bars, the amount of the public health market that these guys sort of take as it stands at the moment. You will see a number of the chemical companies are not even in the vector control market; they believe it's too small a market potentially to actually be there. And we have a very large generic sector, which doesn't necessarily do any R and D work and is not going to innovate and put new products out there. So we really have to work with these small numbers of companies who really are going to be the source of some of the innovation for the new programs.

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Again, just summarizing really what I've said to give you the timeframes. In the 1940s, we had the organochlorines such as DDT and lindane coming through. Those are the red bars. In the 1950s we had the organophosphates, coming through into the 1960s with the carbomates; that's the yellow and the blue set of boxes. Toward the end of the 1960s, early 1970s, we had the pyrethroids coming through in their various guises. And I'm calling etofenprox here a pyrethroid even though I know it's a pseudo-pyrethroid there. But really the main string insecticides stopped coming through into the market in the 1990s, and we've had nothing else since. We really do have to make sure that we now fill this pipeline and we bring new compounds through.

Now, if we're going to do that, clearly we have to work with a market that is big enough for the chemical companies to be interested, or we have to change the paradigm so that we can, by putting funding into their programs, stimulate them to go for a market that would maybe not be big enough for them to look at without our intervention. So again here, this just gives you the split between the indoor residual sprays, the space sprays and the RTM markets. If we've got compounds that work for all of those, we're more likely to get industry to produce an insecticide that will go through into that. And if things are growing, and this is just the same data that I

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showed you but put in a different way, win/win we will be able to work with the companies in a market that is expanding slightly rather than one that has been contracting for many years.

Now, to do that we maybe also need to get buy in from the crop side because again, everybody who's worked in this business knows that the agrichemical companies produce insecticides but first of all primarily for the crop side of the market, and only then do they look at what they're going to provide for the public health market. So we're not saying that we have to just work with the public health sector here. What we are saying is that we will work with industry to see what we can bring over from the crop side to the public health side, and we can work with them on innovative products that may work for both. But we can also work with them for innovative products that may only work for the public health side. We also understand that we may need to put more funding in if that is the latter case.

But if we can something that will break pyrethroid resistance, what industry is telling us is that there is plenty of pyrethroid resistance out there in their crop market. And therefore, a product that breaks resistance in mosquitoes, which also works to break pyrethroid resistance in some of the major crop pests, would actually be of great interest to many

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of the agricultural producers. So I think there are plenty of ways of attacking this problem, and there are going to be an awful lot of discussions going on over the next few months, as we decide which products we should really be going for.

So this is really vector solutions through partnerships. This is not me who is actually going to turn out this program, this is a whole raft of partners. We have at the end set of this program five academic partners working together, and we are looking to bring others within that network, whether with good ideas to be brought through, we're clearly needing to bring the chemical companies in. And again, we're not looking to work with any one chemical company in isolation. We want to be working with all the different chemical companies if we can do so, to stimulate them all to come through with new solutions.

We've already been thinking about this for quite some time, and this is a workshop that was held four years ago now. And those of you who are close enough to the front to be able to see, I hope you'll recognize that within that crowd of people there, we not only have the academics and the chemical companies represented, but we also have the operational control programs, in this case, for Mozambique, for Swaziland and South Africa. But we are very much wanting to work with the operational people to ask the question, "What is it that you

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want? What would make your life better in terms of having a much better compound? Is it an indoor residual spray that would last 12 months rather than three months? Is it a longer-lasting net that would last for four years? Is it a mixture on an indoor residual system or a net that will avoid problems that you may have with resistance?" And as we're actually bringing these solutions forward, we will be testing them in an operational setting, working with the operational people to make sure that we're getting it right. So this partnership extends again, not only through the academics and into industry, but with the operational people whose job it is to actually try and make sure that we really do reduce transmission at the end of the day.

So we've got a public/private partnership, coordinated by Liverpool, and I get the job of attempting to do that. We've got the London School of Hygiene and Tropical Medicine working with us. We've got the NRC in South Africa. Our funding is coming from the Bill and Melinda Gates Foundation. We've been talking to WHO in its various guises as AFRO, WHOPES [misspelled?] and others to make sure that they're onboard as well, so we don't reinvent the wheel with them. We've got Colorado State University, and I couldn't manage to lift off the web the University of California, Davis. I'm afraid they haven't got their logo on their just because I couldn't manage

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to actually sort of pull it out from there.

Working with the Foundation right now we've got the next six months to really gear this program up. We've got to put the portfolio system in place that will allow us to look, in a transparent way, at all the possible proposals that are coming in from industry, with academic partners, to make sure that we really do get the best products into our pipeline and we stimulate those sort of coming through. We want this to be a win/win situation, and we've modeled ourselves to an extent on the medicines for malaria, sort of that system, where we get input from the whole community in terms of what we want and our outputs at the end of the day, we hope, are better controlled to sort of take through.

So this is the MMV portfolio in 2003, and you can see that they've gone from exploratory phase right up to actual production, with the different compounds that they've put into their pipeline. I hope in 12 months time I'll be able to stand up at a meeting like this and say, "This is the pipeline that we have now for our set of systems. These are the products that we've got waiting to come into the pipeline, because we've got so many good ideas out there, but we can't fund all of them at once." That would be a really great position to be in.

As I've said, we want to look at all stages within this sort of set, so the so we're going to look at the different

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phases, three from development of current molecules that are through there, three to improve formulations to actually sort of take the system out there. I hope we'll be able to get some quick wins through that system, potentially on the formulations side, but we do need to make sure that we're feeding that pipeline from further down industries sort of system, because if we only concentrate on one end of the system, then we're clearly not going to get this to work in the format that it needs to.

There are huge benefits then to be had if we get this right, because if you look at the economic modeling of what's been put through, and you ask the question, is it a good idea, for example, to go through with indoor residual spraying, and the huge arguments that have been going on between the DDT and other insecticides there, one of the real big reason that people still want to use DDT is that where you've got year-round transmission, you can get away with one round or at most two rounds of DDT spraying in a year rather than four rounds of anything else. If we can come out, for example, with an IRS formulation that lasts as long if not twice as long as a DDT formulation, then we can change the whole dynamics of that indoor residual sort of spray systems. So clearly, by going down that track with formulation, we've got big gains to be had up front.

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And that changes the dynamic then in terms of the role that vector control could play within a joint drugs and vector control system, and ideally when you've got the vaccine there, within a joint vaccine, drugs and vector control set, so that we really do start reducing the transmission of many of these vector borne diseases that at the moment we're not managing to do with any one system on its own. I think everybody now realizes that we need all three legs of that stool to really name a big sustainable impact on disease control. And at the end of the day, this is really what this program is about.

We've based what we're doing on a whole raft of things, including some of the big resistance trials that have been going on in Mexico. So we know in exactly the same way that monotherapy with drugs is bad news, that monotherapy with insecticides is bad news. If you just go in with one insecticide and you keep using that insecticide until it fails on you, and then you go on to the next one, there is nothing more certain than you will lose your insecticides at a far more rapid rate than you can feed your pipeline. So what we would like to do is to make sure that we understand what is the best way then to employ the tools that we're actually putting out there.

And on the other side of the consortium arm from the new insecticides, we have the improvement to control tools.

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There are four projects that are pre-selected already in that area. Three of those are clearly interconnected. So the lead one, if you like, is a decision support system linked up with a geographical information system that we hope will allow communities and control programs to ask the question of the system, "What is the best way for us to undertake our vector control?" And to be able to do that, we need to make sure that our GIS systems are working extremely efficiently and we've got good information going into that, because if you base your decisions on very poor data, there's nothing more certain than you will make bad decisions. So we're looking at how we can feed that GIS platform with good information. So one of the other projects then is a very high three put [misspelled?] population monitoring scheme for the mosquito vectors to say what kind of resistance do we have, what level of infection to we have in those populations, and what kind of species do we have, because sometimes, as many of you know, it's not easy just to know a collection to be able to tell what you've got in there.

And the third arm of this sort of project that is moving into that sort of set is a modeling system. So there are lots of models out there at the moment for malaria and for dengue. We want to work with the best of those and interface those with our GIS sort of platform. And the two together

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should then allow us to produce the decision support system that we require. So although these are distinct projects in their own right, they should all feed each other to make sure we get the best sort of system after there.

And then the final one is a very much smaller project, which is to do with the quality control of what is going out there. So this is a pyrethroid quantification system that we can use in Africa in the field to make sure that we know how much insecticide is sitting for example on nets or walls once we've actually sort of put those out there, so that we know when to repeat, or that we've got a quality control system for when things are produced and actually put out there in the market.

So what I hope we can do at the end of the day is actually get a reduction in disease transmission coming through without the huge surge in resistance that potentially has come through from different areas. This is very recent data from southern Africa, where we clearly got pyrethroid resistance coming through; that's where you've got the lightning sort of arrow there within this sort of set, superimposed on top of transmission in three areas. The highest level of transmission in one particular area, and it doesn't take much guessing to realize that the area that we got the pyrethroid resistance was the one where the transmission spikes. And then we were forced

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in that case to go back very short term to DDT and then to some other insecticide classes to bring that transmission back down. We would much rather be in the position where we can actually get control, get the right decisions made, before we get this increase in transmission, that we don't have to wait for more children to die before we realize that our vector control is working; that we actually put a system in place that actually gets that running.

I'm told I've got probably now about 15 seconds; I think that's where I'll finish. I think the next one is just a picture of the mosquito. The idea is clearly to control these beasts. I've got very fond of them over the many years we've been working with them, but I'm sure we'd all like to see less of them. And I hope the program that we're putting in place will allow us to do that over the years, and that the community is going to work with us to make sure that we make the best use of what is a huge opportunity we as a community have now been given. And I look forward to working with all of you to try and make that a reality over the next few years. Thank you very much. [Applause]

LIZETTE KOEKEMOER: Thanks, Janet. I think we've got maybe time for one question; then we'll go on to the one. Yes.

[Speaker off microphone]

JANET HEMINGWAY: Okay. I think the first step that we've got

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in terms of actually working out what we want to put into our portfolio is working out exactly where our boundaries sit, because although the headline figure of \$50 million may sound like a very big figure, we all know that actually putting a new molecule into the system, or even taking a molecule that's partway down the system right the way through is a fairly expensive business. And so, I think step one of our program is to say, "Are we going to take larvacides into our portfolio? Are we actually going to look at the technology that's used to spray? Where do we put those boundary lines? Are we going into biopesticides? Are we going into a whole raft of areas?" And I think we need to sort of try and sort those boundaries fairly rapidly, and I think that will be happening over the next couple of months, and we'll try and get that information out there.

So I don't have a simple answer for you now, because I think we have to work together first of all to actually say, "Is this within our sort of set?" I think it's certainly not right within the core of our set, because at the moment I know of no mainstream control program anywhere that is actually using larvacides on their own. I know they're certainly using them in combination with other sort of systems, but I think we have to ask the question, "How much can we really get into that pipeline?" And I can't give you a simple answer there yet.

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LIZETTTE KOEKEMOER: Thanks very much. For the next talker, Josiane Etang. She's going to talk to us about a study done in Cameroon, about the genetic diversity of insecticide resistance.

JOSIANE ETANG: Does this work? Okay, thank you for giving me the floor. This presentation is focused on genetic diversity of insecticide resistance of *Anopheles gambiae* from Cameroon. *Anopheles gambiae* and *Anopheles arabiensis* are the major malaria vectors in Cameroon. All of our most studies focused on *Anopheles gambiae* because it is widespread and the most adaptative vector.

So there is a growing attention to [inaudible] susceptibility in *Anopheles gambiae* since insecticide treatments were adopted by the malaria control program to prevent malaria in Cameroon. So this picture is showing the two sites where we carried out the investigations on insecticide resistance. You see in the tropical areas and in the [inaudible] areas [inaudible] the eastern part of the country and the western part of the country where we had not yet carried our investigations.

This picture is showing the susceptibility statues to DDT and pyrethroids. You can see in the modern part [inaudible] we found DDT and pyrethroid resistance. In the cotton fields located in the northern part, we had permethrin

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and permethrin resistance. In the coastal area and inland equatorial area, we had DDT and [inaudible] to high-level permethrin resistance. The situation is a little bit different in the western part, where we found very high DDT and permethrin resistance with significant increase in knock down times.

So when we carried out the susceptibility test, we demonstrated that there is resistance. Then we went to molecular analysis to see if there was kdr mutation, which is widespread in West Africa. But none of our specimens displayed kdr mutation. Then we went to biochemical analysis to see if there is something, and then we analyzed [inaudible]. We also analyzed the [inaudible]. As you can see in this study, esterase resistance is found in only four populations that we pointed out here. But [inaudible] was only found in the [inaudible] population and GST was found in the [inaudible] population.

So on the previous result, we thought that metabolic-based resistance was the main mechanism observed in Cameroon. And then we wanted to see if it is the case in all the areas. Then we chose three new sites named Foubot in the western part, Bwala [misspelled?] in the coastal part and Campo in the coastal south part. In this table, we show the frequency of kdr mutation in M and S molecular forms of *Anopheles gambiae*.

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In Foumbot, we found more [inaudible] in the S form. In Bwala, we found just two [inaudible] with the kdr in the M form. In Campo, we found also few [inaudible] in the S form. So for us this is very interesting because it is the first time that this mutation is reported in Cameroon.

We were so excited with that result that we wanted to know if it is the real situation. Then we run the [inaudible] protocol, which was published this year. You can see in this [inaudible], there is mutation is [inaudible]. So in the two lateral columns are susceptible detector. In the two middle columns, you have the first one, detecting the kdr east resistant specimen and the other one detecting the kdr west. So as you can see here, we have most of specimen carrying the west mutation and two or three of them carrying the east mutation. What is very special in this slide is that we found two individuals carrying both mutations. So these results, we were not sure on them, and we sent some specimen to Beline [misspelled?] so that she can make sequencing the DNA.

When she confirmed to us that the two mutations were found in those specimens, so you can have a picture of this difficult situation in Cameroon where in the northern part you find esterase or oxidases [misspelled?], and then maybe you have esterases or GST. In the west you have kdr [inaudible]. So in the middle one you have the two cycles. We think it is

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very difficult suggestion for selective vector controls, because as you can see in the [inaudible], selective vector control is application of specific targeted [inaudible] and cost-effective methods, in combination with [inaudible].

So the National Malaria Program—we think that there is a need to carry out further investigation to see what is the distribution of the two mutations in the country. So I don't know what is wrong with this picture, but for both of these results, you wanted to know if [inaudible] resistance there is a decrease of RTM effectiveness. In the left, you have [inaudible], in the right mortality rates. So here we compared [inaudible] rates and mortality rates between. The [inaudible] laboratories we displayed some [inaudible] individuals with elevated oxidase. We compare [inaudible]. As you can see, [inaudible] there is no significant difference between the two strains. But with mortality rates, we observe a significant decrease of mortality on the [inaudible] strain. This means that if you want to use ITNs as a [inaudible], you reduce the vector density in the population, you will not reach the objective. But if you want to use it as a [inaudible] protection, maybe you can be protected but mosquito will be [inaudible], you must use the mosquito nets.

We had this laboratory result, but we wanted to know in the field what is the real suggestion on malaria prevalence and

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malaria morbidity. In this slide, we present the result that we obtained in Peetwa [misspelled?], where we observed oxidase and esterases. As you can see in April, before we implemented the ITNs, the malaria prevalence in the chief study groups were not significantly different. In July, after two months of net utilization, we observed a decrease of malaria prevalence in children using the [inaudible] treated nets and [inaudible] treated nets. The difference, compared to the control group was not significant. After six months of net utilization, the malaria prevalence began to grow, so that it was no longer different between control and treated group.

In this [inaudible], we have a malaria morbidity in children using treated nets. With [inaudible] treated nets we observed a decrease. But this decrease was not observed with the [inaudible] treated net, and when we compared the treatments, the difference was not significant.

So you can say that there are several patterns of [inaudible] resistance [inaudible] in Cameroon. But there is a need for further investigation on the kdr prevalence and introduction which [inaudible] resistance. We should also assay the impact of resistance when the mosquito is carrying both [inaudible]. And then I think that this kind of mosquito need a gun to shoot so that it can die. So we have to assess the impact of resistance on the efficacy of existing tools, and

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then we need to think about alternative strategies as
[inaudible] for selective vector control.

Here I would like to thank people who contribute to the
investigation. This is the end of the talk. Thank you.

[Applause]

LIZETTTE KOEKEMOER: Thanks very much. It's a very
interesting talk, and I think that you've got an excellent
demonstration of all the complexity that's involved dealing
with resistance. Any questions?

MALE SPEAKER: [Inaudible] that there are two mutations
of the same mosquitoes. And Janet, I think you have to get
your map of Cameroon.

[Speaker off microphone]

JOSIANE ETANG: The sample size? About 100 mosquitoes
[inaudible] sides.

[Speaker off microphone]

JOSIANE ETANG: Thank you.

LIZETTTE KOEKEMOER: Any other questions? Thank you
very much for this interesting talk. [Applause] Our next
speaker also I don't think needs much introduction. Dr.
Fabrice Chandre is going to give us a talk. Thank you.

DR. FABRICE CHANDRE: Thank you. So my presentation
will be about malaria vector control and its interaction with
insecticide resistance and the contrary.

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Despite many recent findings on insecticide resistance mechanism in malaria vectors from Africa, there are two big questions that remain to be much more investigated to my point of view. This is the impact of insecticide resistance and the efficacy of vector control methods, because this has direct operational implications for all national malaria control programs. And this is the possible selection of resistance mechanisms by public health operations, because this has a direct implication and is a necessary step for implementation of resistance management strategies. But I will try to illustrate how it is difficult to answer to this question through some examples we obtained in Phase II and Phase III in West Africa against resistant *Anopheles gambiae*.

Just to remind you that in *Anopheles gambiae* from West Africa is now mutant resistant to insecticide. We have several resistance mechanisms to target site mutations. The kdr mutations have conferred resistance to pyrethroids and DDT, and the Ace-1R mutations have conferred cross-resistance to organophosphates and carbamates. We also saw some population with increases of oxidases and esterases.

So the Phase II consists of small scale of trials that are conducted in experimental huts. These experimental huts are [inaudible] to evaluate the efficacy of treatment on impregnated nets, so treatment for indoors in controlled

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conditions. This is a station from Melanville and also Benin, but there are also some stations in Cameroon and Cote d'Ivoire and in [inaudible].

So this slide shows the mortality of [inaudible] with impregnated nets in two sites from Cote d'Ivoire. In the first site, the compare for [inaudible] was very low, around 0.05, which is [inaudible], and we can consider the population of most susceptible, and on the other side the population of *Anopheles gambiae* where a very strong [inaudible] was strongly resistant to pyrethroid, with a kdr frequency around 0.95. Here you can see that the impact of permethrin on [inaudible] mosquitoes is exactly the same or even slightly higher for the resistant population, but you have 45 mortality for each susceptible population, and 55 for resistant populations. The permethrin impregnated nets killed [inaudible] as well as [inaudible] mosquito.

This slide shows the studies that were conducted in South Benin where the kdr mutation is around 70 percent, and it was to see if the different [inaudible] of permethrin bednets were able to select for increase of resistance among populations. So we compared kdr [inaudible] between mosquitoes that were dead and those that were alive in the experimental huts. And we see that for any [inaudible] there is no selection of increased resistance by permethrin-impregnated

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nets.

[Inaudible] that was done [inaudible] plastic sheeting that were lined on the walls of the experimental huts which [inaudible]. And here we see that treatments is much more efficient against susceptible mosquitoes and resistant mosquitoes too, suggesting that this kind of treatment would select for increase of resistance. In fact, all this data shows that the possible selection of resistance by treatment depends on the vector control [inaudible], and are so dependent on the resistance mechanism. I don't have slide to show all [inaudible], but for example for [inaudible] mutation, the nets treated with [inaudible] increased with frequency of [inaudible]. So this is [inaudible] mosquitoes, which is modified by the treatment in the huts and [inaudible] which is modified by the kind of resistance, which is involved.

So now [inaudible] we met several scale free trials in Cote d'Ivoire. The first one was done with cyhalothrin permeated nets in eight villages with four treated villages and four control villages. This area was chosen because the population of *Anopheles gambiae* was strongly resistant to pyrethroids with a kdr frequency of less than 0.85. This high resistance level was mainly due to the selection pressure, which is very strong due to the agricultural strong insecticide use to protect the cotton. But despite this very high

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frequency of resistance among the population, in the treated villages we obtained a protective efficacy against malaria morbidity of 56 percent and with a reduction of malaria transmission around 80 percent, which is similar to what is observed in the susceptible area.

Now what about the evolution of kdr frequency between both villages? Of course, the kdr frequency was very high at the beginning, but all along the 14 months that the study was done, we didn't see any impact of insecticide [inaudible] on the increase of the kdr mutation. So there is no increase of resistance there in the treated village as compared to the control village. And this was also observed [inaudible]. So in areas where there is a strong external selection pressure on population of *Anopheles gambiae*, it seems that insecticide treatment did not increase the resistance in the mosquito population.

So this was the second [inaudible] trial that was done in Cote d'Ivoire, in the west of Cote d'Ivoire. Here [inaudible] because contrary to the previous one, the kdr frequency was very low, less than 0.1, and the population can be considered as the most susceptible. There were two groups of three villages. Three were treated with long-lasting nets and three villages were untreated nets. So there is no selection pressure by insecticide from agriculture in this

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area.

Here we followed kdr [inaudible] frequency in both groups of villages for 15 months, and you see that during the first six months there is no difference between the treated villages and the untreated villages. At nine months, we begin to observe an increase of the kdr mutation in the treated villages that we do not see in untreated, but after 12 and 15 months, the increase in kdr mutation in the treated villages was significant. So it shows that [inaudible] *Anopheles gambiae* are much mutated to strong selection pressure of, for example, by agriculture. So the ITMs can increase the level of insecticide resistance of one year [inaudible].

So just to conclude, the relationship between vector control and insecticide resistance is really complex. It depends on many things, [inaudible] the resistance [inaudible] that modify the resistance mechanism itself can become a modification of insecticides that are more or less a return to the mosquito. Of course, the mosquito behavior is involved and can be modified by resistance [inaudible] external selection pressure. The genetic structure of populations that [inaudible] the spread of resistant genes, and of course the connection between resistant genes [inaudible] if we have metabolic resistance and kdr, we don't know exactly what will happen. So we need to carry out much more study on this

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aspect. Thank you. [Applause]

LIZETTTE KOEKEMOER: Thank you very much for the interesting talk. Are there any questions? The back?

[Speaker off microphone]

DR. FABRICE CHANDRE: Not specifically in this study, except maybe in that area where you see that the untreated [inaudible] a period of a year where you don't have any resistant mosquitoes in the villages. So the impact of [inaudible] in this case is [inaudible] variation. But of course, in all the Cote d'Ivoire where you have the [inaudible] of kdr, it is more easy to find this [inaudible] variation. When it's very high or very low, the difference is less important.

LIZETTTE KOEKEMOER: Any other questions? Okay. Thank you very much. [Applause]

ETIENNE FONDJO (YAOUNDE): We are going to continue our [inaudible]. I want to bring Nkem Okoye down.

NKEM OKOYE: Good morning. I'll be talking to you on inheritance of resistance and relative fitness of pyrethroid resistance *Anopheles funestus*.

Insecticide resistance in *Anopheles* resistance has been implicated as a major factor that has led to the upsurge of malaria in South Africa from 1990s onward. And this was because the DDT was being used to control malaria in South

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Africa, and when this was stopped, this vector was reintroduced from areas where it had been eradicated before. And this discovery has led to the necessity of incorporating a resistance management strategy into the formal malaria control policies in the affected provinces. And these resistant management strategies rely on the assumption of reduced fitness in vector populations. And the principle is that the resistant genes will tend to drift out of the vector populations in the absence of insecticide selection pressure.

Now this figure, we've seen it early. This is just to show that malaria cases on South Africa, and you can see that from 1996, when DDT was withdrawn, the malaria cases shot up.

The first aim of this table was to study the mode of inheritance of pyrethroid resistance genes in *Anopheles funestus*. These strains of *Anopheles funestus* were used for this study. The first, FUM0Z-R is *Anopheles funestus* from Mozambique. And this strain is [inaudible] to one percent mortality when exposed to permethrin. And the second strain is FANG, which is *Anopheles funestus* from Angola. And this strain is really susceptible to permethrin. For the inheritance study, reciprocal crosses were carried out both the susceptible and the resistant strain. The FI progenies were obtained. But FI progeny was then exposed to 0.75 percent femitry [misspelled?], and the mortality was recorded at five-minute

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intervals, and also at 24 hours post-exposure. Some of the F1s were now backcrossed to get the resistance and the susceptible strains, and the progeny obtained from this was also exposed. Furthermore, we went further to cross the F1 progenies among themselves to get an F2 [inaudible]. And the mortality level there was also recorded.

This graph shows the time mortality response for the F1 progeny when the risk [inaudible] backcrosses were carried out. And the results show that the two F1 progeny responded alike, so 0.75 percent femitry. And this indicated to us that there was no sex-linked factors that was involved in the inheritance of these genes.

This second graph is shows the mortality level when the backcrosses to the susceptible strain were exposed to permethrin. And the results shows that the level there is not like that of the resistant strain, and this shows us that it's inherited in an incompletely dominant manner. This also stands to strengthen our belief on the first graph, and this was obtained when the F1 progeny was backcrossed to the resistant strain.

From these results, we can say that the resistance was inherited in an incompletely dominant manner. And from the backcrosses we've been doing up to date is suggesting that this resistance is monofactorial. And in addition, we have been

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monitoring the resistance level in the baseline colonies from which we got the resistant strain, and we've found that the resistant strain has been stable in the absence of insecticide selection pressure.

The second aim was to establish the related fitness of insecticide resistance of insecticide-resistant *Anopheles funestus* when compared to their fully susceptible counterparts. For these studies, we used 30 families from both the susceptible and the resistant strains. And these were followed through from the egg stage, from egg laying to adulthood. And the parameters that were studied included reproductive characteristics such as the fecundity and the fertility. And we also looked at the sex ratio, the survivorship in each stage and also the developmental time, the time it took for the different studies. And these were compared between the resistant and the susceptible strain. And it was also statistical methods were used to find out if it was significantly different.

This figure shows the time spans in the life stages, and we found that in the larval and the pupal stages, there was no significant difference between the two strains. But in the egg stage, the susceptible strain took a slightly longer time than the resistant strain. We also found out that for the reproductive characteristics, they were identical in both

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strains, but for the resistant strain, we recorded a higher number of eggs hatching. And the resistant strain also produced a higher number of female progeny. For the survivorship, we found that they were identical for both strains.

From these results, we can say that there is not fitness cost that's present in these *Anopheles funestus* populations. And these become very important when designing an effective resistance management strategy to combat resistant *Anopheles funestus* populations.

My acknowledgements goes to Professor Maureen Coetzee, Professor Richard Hunt, Dr. Basil Brooke, Dr. Lizette Koekoemer, and all staff and students of Vector Control Research Unit, funded by Wellcome Trust. Thank you.

[Applause]

ETIENNE FONDJO (YAOUNDE): Thank you very much for that presentation. Are there any questions to ask?

[Speaker off microphone]

NKEM OKOYE: Yes, we're actually still working. We're still backcrossing and we want to check the fitness when we're done, not unlike the sixth generation or something.

MALE SPEAKER: [Off microphone], so maybe you are comparing vectors, which are [inaudible], and maybe it's not only [inaudible].

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NKEM OKOYE: What happened here was that for the resistant strain, we tried several times to get a susceptible strain out of it, but it was not possible. So we had no choice but to use an [inaudible] susceptible strain.

[Speaker off microphone]

NKEM OKOYE: I didn't get you. [Laughter]

ETIENNE FONDJO (YAOUNDE): Okay. Thank you.

[Applause] The next presentation is from Manisha Kulkarni. Manisha. Okay.

MANISHA KULKARNI: Thank you. I would like to talk today about a method that we've developed for high-throughput kdr screening of mosquitoes and applications to *Anopheles arabiensis* populations in Tanzania, where we've observed some tolerance to permethrin.

As we know, with efforts to scale up insecticide treated net use in Tanzania and other parts of Africa, there's increasing concern about the development of pyrethroid resistance. And the knockdown resistance mutation is one of the major mechanisms. As we've seen before, there's a change from leucyl-phenylalanine observed in West African populations of *Anopheles gambiae*, and the serum mutation observed at the same codon in populations in Kenya. The kdr allele is completely recessive, and this makes it difficult to detect by conventional bioassay methods unless it's present at high

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frequency.

As we've seen through models of the spread of drug resistance, there is a potential for a very rapid increase in the frequency of resistance in the population from undetectable levels to levels that can quickly result in control failure. And bioassays, which cannot detect the heterozygous proportion of the population are less sensitive at low levels of resistance, since these heterozygotes are more numerous than the resistant homozygotes. Direct genotyping therefore provides a more a more sensitive method for detecting low frequency resistance and can facilitate early detection of resistance in order to avoid control failure.

We've seen different methods that are available for kdr genotyping; however, these have certain limitations when being applied to large numbers of samples in routine kdr monitoring, as is the case for national resistance monitoring programs. Multiplex PCR has certain limitations in that it can be quite time consuming and uses toxic reagents [inaudible]. The PCR/dot blot method is still somewhat time consuming and scoring can be subjective if done by eye or expensive if automated equipment is to be used.

And for this reason we developed a rapid high q-put method that can be used locally in Tanzania for kdr screening. And this is an SSOP-ELISA method, based on that developed

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Allfrangis et al for detection of drug resistant mutations in plasmodium. This method using a single step PCR, followed by detection of the genotype using sequence-specific [inaudible] nucleotide probes in an ELISA format. And it's proven to be rapid and that we can produce results for more than 100 samples in a single day from DNA extraction through to the end of the ELISA. It's convenient in that it uses a 96-well plate format for DNA extraction, PCR and ELISA, allowing easy and rapid transfer of reagent samples between plates. And it's cost-effective, amounting to roughly 1 GBP or 2 USD per sample tested for all consumables reagents. The use of primers developed by Kolaczinski for the dot blot method and [inaudible]. And you have three sequence-specific probes for the LY type, the F West African kdr and the East African kdr, which are labeled with digoxigenin.

So very briefly through the methodology step by step. We start our with our samples, 88 samples per plate, and these can be individual mosquitoes, either whole or in part. We've also used mosquitoes that have been processed for us [inaudible] ELISA, so head and [inaudible] ground up in a grinding buffer. DNA extraction is then done directly in a PCR plate using the rapid salt trace EDTA method, and the resulting DNA extract is pooled into a new PCR plate. The pooled DNA is then used in our PCR reaction, producing a biotinylated PCR

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product. The PCR product is then diluted and denatured, and we add this to plates that have been coated with streptavidin, allowing binding of the PCR product. And after addition of a dig labeled probe solution, we have our first incubation for one hour at 53°C. Following this is a series of wash steps of low and high stringency, taking approximately half an hour, after which we add our proxase-labeled anti dig solution and have a final incubation for one hour at room temperature. After a final wash step, we add our TMB substrate, which produces a blue color change in positive wells. This can be easily read by eye or we can stop the reaction with sulphuric acid, which produces a yellow color change, and this can be read on a plate reader. The OD can be read easily at 450 nM.

So we optimized our reaction using a set of control mosquitoes corresponding to the different genotypes. This shows the sensitivity of ELISA, with the three different columns—I hope you can see clearly—containing the three different probes of L, F and S and the yellow rows are those which had a positive reaction. So on our control we use the LF-70 strain of *Anopheles gambiae* which is homozygous susceptible. We've also used the dondertha [misspelled?] strain of *Anopheles arabiensis*, which works equally well. It's not shown here, though. The F control in row two is our V kdr strain of *Anopheles gambiae*, which is homozygous for the West

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African kdr. And we used the RSP strain of *Anopheles gambiae* as or S control, as it's homozygous for the East African kdr. In rows four and five, we have a [inaudible] of two mosquitoes, one susceptible and one resistant, which creates an artificial heterozygote which is easily detected by both probes. And in rows six and seven, we have a further dilution to the mutation, showing that we can pick up one heterozygous mosquito in a pool of two mosquitoes. And of course row eight, [inaudible] control.

So following initial optimization, we established a technique [inaudible] program research center, which is located in [inaudible] Tanzania, shown in the little square at the top of the map. And we've since done further optimization with [inaudible] to mosquitoes collected from various field studies. Initial applications were on *Anopheles arabiensis* from the lower [inaudible] field site. And this is an area where we have ongoing characterization of insecticide susceptibility status of *Anopheles* and QX populations as well as evaluation of insecticide-treated materials.

So in this area, in WHO tests between 2004 and 2005, we've seen more and more [inaudible] of about 92 percent, which is a level that requires further evaluation according to WHO standards. So we applied our SSOP/ELIZA technique to survivors and dead mosquitoes from this bioassays in order to determine

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whether there is a kdr component to the tolerance we've observed. We also applied it to survivors and dead mosquitoes collected from experimental huts, where permethrin-treated materials were used to look for any selection of the kdr mutation. And finally, we applied it to mosquitoes collected from the longitudinal monitoring project in a village where we were measuring inoculation rates.

So this small table shows the results of our initial applications, with the numbers tested and the number that we found kdr positive. What we found is that in our [inaudible] field site, where we've seen phenotypic tolerance or resistance to permethrin in our susceptible retest, there is no involvement of kdr. And the same was found for the mosquitoes collected from the experimental hut from the same area. What we did find, however, is that in our longitudinal monitoring project in a village with no selection pressure from ITN or agricultural insecticides as far as we can tell, there were two mosquitoes that tested heterozygous for the East African kdr mutation, and these samples are awaiting further confirmation by sequence analysis.

The lack of correlation between genotype and phenotype as far as permethrin tolerance is concerned indicates another mechanism of resistance. We need ongoing work to characterize enzyme-based mechanisms. What's interesting is that the low

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frequency of resistance observed in village with no selection pressure may indicate a low level of this resistant mutation occurring naturally in the population, as we've seen in western Kenya before intervention with ITN.

So while KDR genotyping has been the main application of this method so far, I'd like to mention a couple of other applications. And the first is adaptation to other point mutations that confer resistance, and we're currently optimizing the ACE-1 insensitive acetyl cholinesterase assay for use on field mosquitoes. The primers [inaudible] develop two specific probes for the glycine monotype [inaudible]. We also envision application to other mosquito species, including [inaudible], which as we know is a widespread, nuisance-spreading mosquito that resistance in the species has certain implications for ITN use and therefore malaria control.

In conclusion, the SSOP/ELISA provides a rapid method for kdr screening that's been established at the joint united program center in order to assist in order to assist national resistance monitoring efforts. And while kdr does not seem to be responsible for the permethrin tolerance that we've observed so far in our field specimens, we want [inaudible] work involving [inaudible] bioassays, which we hope, will provide a clearer picture of this in the future. Thank you. [Applause]

ETIENNE FONDJO (YAOUNDE): Thank you very much for

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this presentation. Any questions?

[Speaker off microphone]

MANISHA KULKARNI: Yes, that's what we intend to do.

[Speaker off microphone]

MANISHA KULKARNI: Especially since we're doing DNA extraction in a plate format, we do need to pay quite careful attention to this. It's just a matter of careful handling of mosquitoes and cleaning of instruments between handling the mosquitoes.

[Speaker off microphone]

MANISHA KULKARNI: We did this on control mosquitoes of the strains that I presented here, which match with those of multiplex PCR. We haven't compared it to the dot blot method, though, or the hola [misspelled?] technique.

ETIENNE FONDJO (YAOUNDE): Any more questions? Okay. Thank you. [Applause] Now we'll hear from Samson Awolola.

SAMSON AWOLOLA: Thank you. I'll be presenting on dynamics of permethrin resistance in a field population of *Anopheles gambiae* in southwestern Nigeria.

The use of insecticide treated nets and indoor residual spraying are major components of malaria control, especially in Africa. In spite of the effectiveness, resistance remains a challenge. The problem of resistance becomes so clear when you look at it [inaudible]. More than 80 percent of insecticides

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are not for public health programs. And from a historical perspective, you understand that the failure of previous malaria control programs which partly contributed to vector resistance. Now what you have, and therefore there's a need for us to guide what you have as insecticide.

Pyrethroid insecticides are the only class of insecticide approved for treating nets today. And to make the issue complex, resistance has emerged in the major malaria vectors across Africa. Resistance [inaudible] complex, especially *Anopheles gambiae* [inaudible] is west and east Africa and [inaudible] in southern Africa. [Inaudible] well established, and has some people talking about [inaudible], for *gambiae* in West and East Africa [inaudible].

In 1999 [inaudible] there was little is known about the susceptibility status of malaria vectors to this class of insecticide. At that time, we carried out a study to provide information on this mosquitoes [inaudible] concern. And [inaudible] at that time was fully susceptible in our studies [inaudible], permethrin, deltamethrin, larndacyloathrin and DDT, because of the close [inaudible] DDT has with the [inaudible]. By 2001, we established and published resistance to pyrethroid insecticides in this mosquito population. And therefore the aim of this [inaudible] to show what has happened in the mosquito population over this period of time.

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This [inaudible] in West Africa, and this is the position of insecticide [inaudible].

Now rainfall is a major factor [inaudible]. So the pattern of rainfall in southwestern Nigeria, this is what you find. You have the early rain, the late rain, the early dry season and the late dry season.

And so [inaudible] of this study, what we did to collect adult mosquito [inaudible] to identification of the [inaudible] and the kdr is also carried out in the laboratory.

At the same time, we wanted to see what was happening [inaudible].

Now, for the period of the study, [inaudible] adult mosquito collected, [inaudible].

Now, as we look at the dynamics of the population, we find that two peaks, the early rainy season from 2001 to 2004, you see two peaks starting at each year. And that is not surprising because [inaudible].

[Inaudible] that's what you find in the mosquito [inaudible]. And these are mosquitoes that were here [inaudible]. You find [inaudible] a sharp increase in the resistance level [inaudible] and that fell down to [inaudible].

[Inaudible]

[Inaudible]

[Inaudible]

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In summary for this table I would just want to say that [inaudible] not find much increase in resistance level from 2001 to 2004. The kdr was found only in the molecular S form; the M form possibly has a different resistant mechanism, possibly metabolic resistance. How long will this resistance gene last in the population? We don't know. But what will contribute [inaudible] the molecular M form. [Inaudible] but this is what we try to find. Does this actually support the theory [inaudible]?

I just want to thank the University of the Witwatersrand, to which this study was initiated. The VCRU NICD, the ANVR/WHO-AFRO, WHO/MIM-TDR Grant, and the Wellcome Trust Grant. Thank you. [Applause]

ETIENNE FONDJO (YAOUNDE): Thank you very much for this presentation. Some questions?

MALE SPEAKER: You have said at the beginning of your speech that in 1999 it was a fully susceptible population. And how do you explain this sudden [inaudible] increase of resistance between '99 and 2001? Because it's a short period of time.

SAMSON AWOLOLA: Thank you. In 1999, when this study was started, we did not find any resistance. Not that there was no mosquito with resistance to insecticide, but [inaudible] no resistance. That's what we mean by no resistance

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[inaudible].

ETIENNE FONDJO (YAOUNDE): Thank you. I would like to know at that time if you made the PCR to know if there was [inaudible] or not because if there was [inaudible] supposed to be susceptible, so if you just carry out the assays and end up with [inaudible] mortality rates you can't know if there is kdr or not.

SAMSOM AWOLOLA: Thank you. The [inaudible] samples tested [inaudible] kdr, and [inaudible] was submitted that [inaudible] kdr. We found that in South Africa. [Inaudible] so that all our samples are submitted to kdr tests.

ETIENNE FONDJO (YAOUNDE): Yes?

[Speaker off microphone]

SAMSON AWOLOLA: Thank you. I didn't want to take too much time telling story about the study sites, okay? Because at that time of the study, we carry out [inaudible], there's not much of a culture of using insecticide. But then [inaudible] a very new label, very, very new label.
[Inaudible]

ETIENNE FONDJO (YAOUNDE): Last question? Okay.

[Speaker off microphone]

SAMSON AWOLOLA: Okay. Thank you. Like I said initially, it's too difficult to really see [inaudible] it's a complex thing to establish because you do not know what the

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other factors are [inaudible]. But the use of [inaudible] the sustained impact on resistance. But the point is this: when all these factors interact toward [inaudible], the impact on resistance [inaudible].

ETIENNE FONDJO (YAOUNDE): Okay. Thank you very much. [Applause] Now we are inviting to the floor Mr. Mueller.

PIE MUELLER: My title of the talk is "Microarray Analysis of Genes Mediating Metabolic Resistance in a Pyrethroid Resistant *Anopheles gambiae* Strain from Ghana."

As Janet has already pointed out to you, pyrethroid-treated bednets are the mainstay of malaria control in sub-Saharan Africa and are to date the best insecticides available. But emergence of pyrethroid-resistance threatens to compromise the successful use of insecticide-treated materials.

Now talk sites insensitivity, there are a group of three enzyme families that play an important role is metabolic resistance. The cytochrome P450 monooxygenases, which are thought to play a role in pyrethroid resistance, as well, to a lesser extent, carboxylesterases and glutathione S-transferases. Now although we know that these families are involved in insecticide-resistance, we know very little about the vital members of these families, and therefore our aim is to study more in detail what's the contribution of each gene.

Now up to date, we have some information on permethrin

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metabolic resistance. Measurements have been done, or I should say elevated transcript levels have been measured in a permethrin-resistant strain, RSP from western Kenya. What has been shown is that the GSTe2 was highly overexpressed. GSTe2 has been found in a number of other species and strains and is clearly related to DDT resistance, but its mechanism in permethrin-resistance warrants further research. Also it has been found that cytochrome CYP6z1 and CYP325a3 is related to insecticide-resistance in that particular strain.

Now you can ask us, for kdr resistance, do we find different mechanisms across different populations? Are there any alternative mechanisms involved? That's very important if we want to address new tools toward insecticide resistance monitoring. It will be important to know what we are looking for. So in this study, we measured constitutive or the basal gene expression patterns between a susceptible standard strain and a resistant strain from West Africa.

The resistant strain is originated from Odumasy in Southern Ghana and is resistant to permethrin. Mosquitoes were collected in the field, and in the lab they were exposed on a regular basis to 0.75 percent permethrin in WHO test tubes every one hour. The survival rates of both females and males was fairly high. Our reference strain was the Kisumu, originating from East Africa, and is completely susceptible to

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permethrin.

Now, to look at expression levels between these two strains, we used a microarray approach. We extracted RNA from the resistant strain and susceptible strain, amplified the mRNA in vitro and then labeled the targets either with a red label or a green label. And those two targets were cohybridized together to what we call the detox chip, which is a microarray containing 230 gene fragments. Now among these 230 gene fragments, there were the cytochrome P450s, the carboxylesterases, the glutathione S-transferases and some other genes as well as various control genes. Now the idea is, when you cohybridize these two probes that are labeled either with the green or red label, we get a color signal. If it is yellow, that means you have the same amount of transcripts from both targets, meaning that the transcription level was equal in both samples. If it is red, then it was overexpressed in the red, and green, vice versa. Now on the chip we had also four replicate spots. That means each spot is replicated four times, which gives us a better measurement.

Now, the experimental design looks as follows, with two different independent experiments. In the first experiment, we compared adult females from the Odumasy strain with the expression levels of adult females from the Kisumu strain. And in the second experiment, we did the same thing, but with

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males. In each experiment, we extracted RNA from a batch of 15 adult mosquitoes, so we had three biological independent replicates. For each biological replicate, we also had two technical replicates, which means that we extracted and amplified RNA from one batch of 15 mosquitoes and split them into two alycorts [misspelled?]. One alycort [misspelled?] was labeled with the green dye and the other alycort with the red dye.

This box, which is often called a volcano blob, shows the summary of the full change of expression when you compare resistant females with the susceptible females. So on the X-axis you have the Log₂ fold change, and on the Y-axis, the *p*-value, which is related to the gene wise comparison for each gene. So each dot represents one gene on the table. Now, on the X-axis, zero means equal expression levels in both strains. If you go to the right, that means over expression in the resistant strain. And if you go to the left, that means over expression in the susceptible strain. Now the higher the number on the Y-axis means there was a higher difference, or the significance was higher.

Now because you have a whole cloud of potential genes that are interesting, you use two criteria to sort out genes that are most interesting for us to follow up. The first criterion was the level of significance which we put at 0.001.

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The second criterion was a two-fold change criterion. So we took out all the genes that had a fold change below two folds or above minus two folds, meaning between minus and one on the X scale here.

Using this criteria, we find an over expression in Odumasy females among five genes, and one gene was over expressed in Kisumu female strain. So the five genes that are over expressed are the CYP6z2 and the CYP6m2, which are those that were over expressed most, along with the SOD3b, a member of the suproxidase dysmotase [misspelled?], and a GST as well as an esterase. In the females of the Kisumu strain, we found a CYP6p1 gene to be just over twofold over expressed.

Now, comparing those results from the females with the males, we find three genes that come up in both comparisons, which are two CYP6, CYP6z2 and CYP6m2, along with the SOD3b. If you rank those three genes that are coming up most, we find that the CYP6 genes are those that are the prime genes of interest, because they share the highest over expression as well as a fairly high p-value. Moreover, those two genes are within a cluster that was found within a quantitative traits locus associated with permethrin resistance in the RSP strain.

So to conclude, we can say that we have now two P450 monooxygenases genes that stand out as candidate genes conferring permethrin resistance in the West African *Anopheles*

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gambiae strain, and those two strains constitute a model set of genes that have not been identified so far. And most importantly, it shows that maybe different mechanisms are at work in different strains. That has an implication on vector control and monitoring insecticide resistance.

So, because in the lab the mosquitoes are forced through a bottleneck and they might lose some alleles that are important to insecticide resistance, we are now doing work in the field where we collect [inaudible] females and raise their offsprings and then assess the phenotype of these as female lines and compare them to the expression levels. That will include more potential alleles that occur in the field as well as we would like to have mosquitoes that have more or less the same genetic background.

Now, the ultimate goal is actually to use simple methods such as the hola technique that has been presented just before to develop these assays so that there are new tools out there for monitoring resistance.

I thank you very much for your attention. [Applause]

[Speaker off microphone]

PIE MUELLER: Do you mean the very same chip or we did some trials with *Anopheles arabiensis* because they share quite a lot of sequences with *Anopheles gambiae* and that worked. How far it works for other species that are far away in Gambia,

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we'll see. But the good thing is about this custom-made chip is that we can easily adapt the chip to other species by just replacing the sequences.

MALE SPEAKER: [Inaudible]

PIE MUELLER: That's a very good question. I think that's true, what the powers of this tool is, that you can screen for a whole arrange of genes, so you can dig out genes that are putatively involved with the candidate genes, but then other techniques have to come in to show that these genes are involved in insecticide resistance. And once you're at that stage, then you can make new tools, simple assays that can be applied in the field. Does that answer your question?

MALE SPEAKER: Just a question. Sometimes [inaudible] insecticide resistance, you have [inaudible] expression, which is much higher than two times. So do you think that with your oxidase you can [inaudible] some combination of mutation that includes catalytic properties or oxidase?

PIE MUELLER: If I understand your question correctly, is there a correlation, an interaction between different genes?

[Speaker off microphone]

PIE MUELLER: I think it's very difficult to say what the biological meaning is of a particular fold expression and a function on the gene, but somehow we need to set criteria to look for candidate genes, but we might be following a wrong

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track. Maybe genes that are under expressed are more important than those that are over expressed.

ETIENNE FONDJO (YAOUNDE): Okay. Thank you.

[Applause] This was the last presentation, and to conclude, [inaudible] after the [inaudible] presentation concerning resistance [inaudible] conclusion. But what you consider is that resistance is a change that needs investigation because [inaudible] in each country [inaudible] implementation. [Inaudible] is a big challenge, and scientists must [inaudible] to provide [inaudible]. Thank you very much. [Applause]

[END RECORDING]