



UNDP/WORLD BANK/WHO SPECIAL PROGRAMME FOR
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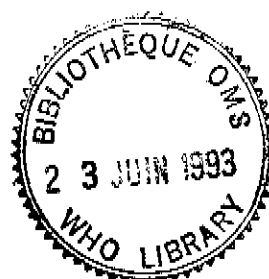
WORKSHOP ON DNA PROBES AND PCR FOR DETECTION OF FILARIAL
 PARASITES IN VECTORS

New England Biolabs, Beverly, Massachusetts, USA
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1. Introduction

The meeting was called to order by the Chair, Dr. Eric Ottesen of the National Institutes of Health. Dr. Ottesen welcomed the participants and urged them to focus on four questions during the meeting: 1) What do we want DNA probes to do? 2) What DNA probes and assays do we have already? 3) How should we use the DNA probes that we have? 4) What do we need to develop for the future?

Dr. Don Comb, President of New England Biolabs, welcomed all of the participants to New England Biolabs for the meeting. New England Biolabs has had several groups working on filariasis for many years, concentrating on cloning genes coding for proteins that may be useful in a vaccine for *Dirofilaria immitis*. Recently, New England Biolabs is focusing on the biochemistry of the parasite.

Dr. C.P. Ramachandran, Secretary of the TDR Steering Committee on Filariasis, welcomed the participants and thanked Dr. Don Comb for his financial

This report contains the collective views of an International group of experts convened by the UNDP/WORLD BANK/WHO SPECIAL PROGRAMME FOR RESEARCH AND TRAINING IN TROPICAL DISEASES (TDR). It does not necessarily reflect the views of TDR/WHO. In the interests of rapid communication it has been submitted to only minimal editorial revision. Moreover, any geographical designations used in the report do not imply the expression of any opinion whatsoever on the part of TDR or WHO concerning the legal status of any country, territory, city or area or of its authorities concerning the delimitation of its frontiers or boundaries.

Ce rapport exprime les vues collectives d'un groupe international d'experts réuni par le PROGRAMME SPECIAL PNUD/BANQUE MONDIALE/OMS DE RECHERCHE ET DE FORMATION CONCERNANT LES MALADIES TROPICALES (TDR). Il ne représente pas nécessairement les vues du TDR/OMS et, en vue d'une diffusion accélérée, il n'a pas été l'objet d'une mise en forme particulièrement soignée. En outre, les noms géographiques utilisés dans le présent rapport n'impliquent, de la part du TDR ou de l'OMS, aucune prise de position quant au statut juridique de tel ou tel pays, territoire, ville ou zone, ou de ses autorités, ni quant au tracé de ses frontières.

support for this meeting and for the last DNA Probes meeting in 1989 in Jakarta, Indonesia. He also thanked Dr. Larry McReynolds of New England Biolabs for organizing this meeting on DNA probes. Dr. Ramachandran stressed the need to accelerate the application of DNA probes in the field. He cited the example of the specific use of *Onchocerca volvulus* probes in the Onchocerciasis Control Program in Africa, where the use of DNA probes to screen vectors is saving the OCP about \$50,000 per year. DNA probes may prove extremely useful in monitoring the effectiveness of control programs. DNA probes may help provide answers to questions such as: Has drug treatment of the human population reduced transmission? How do we know when it's advisable to stop control procedures? How do we know when it's necessary to resume control? The use of DNA probes to screen vectors in large batches may prove to be an effective way to calculate an index of transmission useful in answering these and other questions. These probes will need to be species-specific because many vectors carry more than one species of filarial parasite. The probes must also be sensitive, economical, and user friendly.

Dr. Ottesen and the participants then listed the main issues that should be discussed during the meeting.

- 1) The DNA probes must discriminate infected from non-infected vectors.
- 2) Species-specificity of the DNA probes is crucial in screening vectors because there may be animal filariae carried by the same vectors that carry human filariae. There may also be new species that have not yet been described.
- 3) For monitoring control programs there is a need to detect small numbers of parasites in large pools of vectors. Due to the inherent sensitivity of the polymerase chain reaction (PCR), it will be particularly useful for this purpose.
- 4) The use of DNA probes to incriminate new vectors could be an important application of the technology.
- 5) Do we need stage-specific (i.e. L3-specific) DNA probes to screen vector populations?
- 6) The DNA probe assays should be as user friendly and economical as possible.
- 7) The use of DNA probes to screen clinical specimens such as blood, urine and skin snips may be another important application of DNA probes.

The current state-of-the-art of DNA probes and PCR with regard to each of these issues and others was then presented in reports given by each of the participants. The first four reports are on lymphatic filariasis, the second four are on onchocerciasis, and the last four discuss vectors. Following the reports, all of the important issues were discussed thoroughly first in small groups and then by all of the participants. The recommendations agreed upon by the participants will be listed following a review of each of the state-of-the-art reports.

2. Lymphatic Filariasis Reports

2.1 Larry A. McReynolds--A Summary of the 1989 Jakarta Recommendations and a Review of the Development of DNA Probes for *Brugia*

Summary of the 1989 Jakarta Meeting

Prior to the start of the 1989 DNA Probes meeting in Jakarta, Indonesia, filters containing DNA from many species of filariae were prepared by Catherine B. Poole and sent to all participants who had developed DNA probes for various species of filariae. The various investigators hybridized these filters with their DNA probes and sent the results to Larry McReynolds and Catherine Poole. These filters were coded, so that the investigators did not know the results until they were revealed at the meeting. The oligonucleotide DNA probes for *Brugia malayi* and *Brugia pahangi* developed by S.A. Williams, C.B. Poole and L.A. McReynolds proved to be very sensitive and species-specific. A plasmid probe for *B. malayi* developed by W. Piessens was also sensitive and species-specific. The various probes for *Wuchereria bancrofti* were insensitive and not species-specific. The probes for *Onchocerca volvulus* developed by T. Unnasch, S.E.O. Meredith and others proved to be both sensitive and species-specific.

Recommendations regarding these DNA probes were made and much progress has been made in reaching the goals set at that meeting:

- 1) The development of non-radioactive DNA probes for screening vectors and blood samples was a high priority and today these are now available for *Brugia*, *Wuchereria* and *Onchocerca*. Field studies have been undertaken to test these non-radioactive probes.
- 2) New probes for *W. bancrofti* were recommended and these have now been developed.
- 3) The use of the polymerase chain reaction (PCR) to enhance the sensitivity of detection of L3 larvae in vectors and microfilariae in blood was recommended. Today PCR assays are available for the detection in vectors and blood of *Brugia*, *Wuchereria* and *Onchocerca*.
- 4) Methods for storing and processing materials in central laboratories was recommended. The use of drying for preserving parasite DNA in vectors and EDTA for preserving parasite DNA in blood samples is now in widespread use. New simplified techniques for processing samples are being developed.
- 5) The use of DNA probes to see if strain differences in *B. malayi* correlate with disease state was recommended. Little progress has been made in this area because of the difficulty in developing strain-specific probes for *B. malayi*.
- 6) The use of *Onchocerca* DNA probes to monitor breakthrough transmission in the Onchocerciasis Control Program was recommended and is now part of an ongoing effort by the OCP.
- 7) The use of *Onchocerca* DNA probes to monitor shifts in parasite populations related to disease control in the OCP was recommended and is now in place in the laboratory of Dr. Laurent Toe.

Review of the Development of DNA Probes for *Brugia*

S.A. Williams and L.A. McReynolds developed the first oligonucleotide probes for both *B. malayi* and *B. pahangi* in 1985. These probes were based on the tandemly repeated *Hha* I sequence that makes up 12% of the *Brugia* genome. Using DNA sequence data obtained from repeats cloned from each species, oligonucleotide probes specific for each of the two species were developed. These probes (labeled radioactively) were used in a field study in Indonesia conducted by S.A. Williams and F. Partono. Blood samples collected from infected humans and cats were screened in a double-blind fashion by Williams and Partono using the DNA probes and by Purnomo using classical morphological identification techniques. The two techniques gave 99% concordance in species identification. The study showed the feasibility of using the DNA probes to screen field collected samples. The study also indicated that despite some previous reports, humans were not infected with *B. pahangi* in South Kalimantan, Indonesia. C.B. Poole and S.A. Williams later demonstrated the effectiveness of 100 mM EDTA in preserving microfilaria DNA in blood incubated at 37°C for up to one month. In 1991, S.A. Williams, C.B. Poole, D. Landry and L.A. McReynolds developed non-radioactive biotinylated probes that could detect as little as several hundred picograms of *Brugia* DNA.

2.2 Steven A. Williams--The Use of DNA Probes and PCR to Screen Vectors and Blood Samples for *Brugia* and *Wuchereria*

The report by L.A. McReynolds reviewed the early work on DNA probes for *Brugia* species. *Hha* I repeats have now been cloned and sequenced from several other species of *Brugia* including *B. timori*, *B. beaveri*, *B. patei* and *B. buckleyi*. A total of about 200 repeat sequences have now been cloned and sequenced from six of the ten known species of *Brugia*.

Non-radioactive oligonucleotide probes have recently been developed for *B. malayi* and *B. pahangi* that are end-labeled with fluorescein-dUTP residues. These non-radioactive probes are as sensitive as the original radioactive probes, detecting as little as 100 picograms of *Brugia* DNA (about one-half the DNA in a single microfilaria). The Indonesian samples screened with the radioactive probes described in the previous report have now been rescreened with these non-radioactively labeled probes. The results with the fluorescein-labeled probes were virtually identical to the results with the radioactive probes. Concordance with morphological identification was greater than 99%. The non-radioactive probes were as sensitive and as specific as the radioactive probes.

A detection system for *Brugia* species using the polymerase chain reaction (PCR) has been developed based on the *Hha* I repeat. This technique has been used to detect as little as one femtogram of purified *B. malayi* DNA. This assay has been used to detect a single microfilaria in a 50 ul blood sample and individual L3 larvae. The sensitivity of this system is so great, that it may be

possible to detect one L3 in one hundred mosquitoes or one microfilaria in as much as one ml of blood.

DNA probes for detecting *W. bancrofti* have improved greatly since the 1989 Jakarta meeting. In collaboration with Dr. Suzanne Chanteau of the Institut Malarde in Papeete, French Polynesia, we have cloned a repeat sequence (the *Ssp I* repeat) that is found in isolates of *W. bancrofti* from all over the world. Because the copy number of this repeat is about 300, when labeled radioactively or non-radioactively, this repeat can detect about 500 picograms of *W. bancrofti* DNA. This is not sensitive enough for detecting individual L3 larvae in vectors or individual microfilariae in blood samples. For this reason a PCR-based detection system was developed based on the DNA sequence of this repeat. This PCR system can detect as little as 0.5 picograms of *W. bancrofti* DNA (about 0.2% of the DNA in a single microfilaria). Unlike a previous repeat cloned from Egyptian *W. bancrofti*, this *Ssp I* repeat shows no regional specificity. The repeat has been found in all isolates of *W. bancrofti* tested to date (including a sample of *W. kalimantani* from Indonesia). The repeat is specific to *Wuchereria* and shows no cross-hybridization or PCR amplification with filarial DNA from any other genus.

The *Ssp I* PCR system has been used to amplify a single microfilaria in 50 ul of blood and a single L3 larva in a pool of 50 uninfected mosquitoes. Amplification results on microfilariae in blood and L3 larvae in mosquitoes indicate the assay is semi-quantitative. Further refinement of the PCR amplification technique may result in an assay that can give truly quantitative information. Finally, tests on only the heads of mosquitoes suggest that this method would be more practical when testing pools of one-hundred or more mosquitoes because the volume of the assay can be kept at a reasonable level. Additionally, assaying only the heads provides stage-specific data since only L3 larvae are found in the head of the mosquito.

The *Ssp I* PCR system has also been used to amplify as little as one picogram of *W. bancrofti* DNA added to 100 ul of blood to simulate the detection of circulating or "free" DNA in blood. Research done in collaboration with E. Ottesen and J. McCarthy indicates that circulating DNA has been detected in the blood and plasma of individuals infected with *W. bancrofti* from the Cook Islands. Some of these individuals have as few as two microfilariae per milliliter of blood and two patients have cryptic infections.

A major advantage of the PCR system in all of these assays is that results are obtained after PCR by simply running the products on an agarose gel, staining with ethidium bromide, and looking for a 188 base pair band. Results are obtained very rapidly because DNA labeling, DNA hybridization, post-hybridization washes and complex detection systems are avoided with this method. Results are obtained much more rapidly than with DNA hybridization and the cost is comparable.

2.3 Senarath Dissanayke—A DNA Probe and PCR System for *W. bancrofti*

The development of a DNA probe for *W. bancrofti* had three primary objectives: 1) to identify L3 larvae in mosquitoes, 2) to determine ultra-low microfilaria counts in human blood, and 3) to monitor macrofilaricide screening. Two DNA probes have been developed: 1) Wb 35 which is specific for *W. bancrofti* and can detect about 1 nanogram of genomic DNA when labeled with ^{32}P , and 2) Wb 67 which is filarial specific not species-specific. Use of the probe Wb 35 to detect *W. bancrofti* directly in mosquitoes was difficult due to the large amounts of vector material present. Therefore, PCR primers were designed to amplify the Wb 35 repeat. When used on pure *W. bancrofti* DNA and then probed with a chemiluminescent Wb 35 probe, the PCR system was specific and sensitive. The PCR amplification did not work well on mosquitoes due to inhibitors present in the mosquito. A method was developed to "capture" the *W. bancrofti* DNA from the mosquito extract using a biotinylated Wb 67 probe and avidin beads. After washing and boiling, the Wb 35 primers are then used for PCR amplification. The PCR products are run on an agarose gel, Southern transferred, hybridized with a Wb 35 probe and detected using chemiluminescent reagents.

In collaboration with Dr. Zheng in China, these probes were tested on mosquitoes that were dissected and examined microscopically for L1, L2 and L3 larvae. The dissected mosquitoes were treated as described using the avidin "capture" and PCR/hybridization method described in the previous paragraph. Of 86 mosquitoes found by microscopy to be positive for any of the three stages, 83 (96.5%) were also positive with the DNA probe method. Of 149 mosquitoes found to be negative by microscopy, 3 (2%) were found to be positive with the DNA probe method. Thus, using microscopy as the "gold standard" for comparison, there were 3.5% false negative samples and 2% false positive samples using the DNA probe method. It was concluded that: 1) there was good agreement between the results of microscopy and the DNA probe method and, 2) a method is needed for batch processing large numbers of mosquitoes in a single assay.

Another study in Sri Lanka was undertaken to use this PCR/hybridization detection system to detect filarial DNA in blood. Blood samples were collected in EDTA from microfilariae positive individuals and their household contacts. One ml of whole blood was digested with Proteinase K for 6-12 hours, extracted with phenol and chloroform, ethanol precipitated, and PCR amplified. The PCR products were run on an agarose gel, Southern transferred, and then hybridized with the Wb 35 probe. These samples were also screened microscopically and with a cloned antigen (SXP-1) that is microfilaremia-specific. 90% (17/19) of the microfilaria(+)/SXP-1(+) samples were detected with the PCR assay. 33% (1/3) of the microfilaria(+)/SXP-1(-) samples were detected. Most interestingly, 23% (5/22) microfilaria(-)/SXP-1(+) and 24% (11/45) microfilaria(-)/SXP-1(-) were detected. Overall, the PCR/hybridization assay detected 90% of the microfilarems and 25% of the amicrofilarems. There was no correlation between the PCR/hybridization signal and the serum antibody levels. The

usefulness of this assay in assessing macrofilaricide effects of drugs is being investigated.

Since the Wb 35 repeat is not sensitive enough as a hybridization probe to detect individual L3 larvae or microfilariae, new repeat sequences from *W. bancrofti* are being examined. One solution to the problem of insufficient sensitivity would be to make a cocktail of repeat probes that are all species-specific for *W. bancrofti*.

2.4 Steven A. Williams--Detection of *W. bancrofti* and *B. malayi* in Vectors and in Blood Samples using PCR Amplification Systems

The goals in developing PCR amplification systems for *W. bancrofti* and *B. malayi* were as follows: 1) To reliably detect one infected mosquito in pools of 100 or more uninfected mosquitoes. Such an assay would be extremely useful for monitoring parasite control programs or transmission in regions where the numbers of infected mosquitoes are naturally very low. 2) To reliably detect ultra-low numbers of microfilariae in blood samples. Individuals with very low microfilaremiias are often missed by traditional microscopy methods. 3) To detect circulating or "free" DNA in blood or serum from infected patients. Such an assay might prove useful in identifying amicrofilaremic infections and for screening "day blood" in regions endemic for periodic or nocturnally subperiodic filariae.

The use of PCR to detect filariae in infected mosquitoes has posed a vexing problem due to unidentified substances in the mosquito that strongly inhibit the PCR reaction. In collaboration with Dr. Suzanne Chanteau of the Institut Malarde in Papeete, French Polynesia, we have developed a simple method for processing batches of mosquitoes that eliminates these inhibitors and enables the detection of one L3 larva in a pool of 50 uninfected mosquitoes. In this method, mosquitoes are squashed in a NaOH/SDS buffer. The volume of the solution is brought to one ml with a Tris/EDTA buffer. Activated and fractionated silica particles are added and mixed with the mosquito extract. The silica is then pelleted and washed twice. A few microliters of the silica can then be removed directly to a PCR reaction. This method is the most sensitive and reliable of all the methods we have tried for detecting filariae in mosquitoes.

In reconstruction experiments, one *W. bancrofti* L3 larva was added to pools of 10 to 30 intact *Aedes polynesiensis* mosquitoes. The protocol above was used to obtain DNA for *Ssp I* PCR amplification. A portion (10-20%) of the PCR reaction was run on an agarose gel and stained with ethidium bromide. In each case, the *W. bancrofti Ssp I* repeat band was observed at 188 base pairs. Similar experiments were conducted using only the heads of mosquitoes. Again, a single L3 larva was added to pools of 10 to 50 mosquito heads. Again a positive signal was obtained in each case. We have had difficulty when greater than 30 intact mosquitoes or greater than 50 mosquito heads are used in these assays. Research is continuing to improve the sensitivity of the assay. Our current goal is to detect a single L3 larva in pools of 100 intact mosquitoes and/or 100 mosquito heads.

One important consideration is that 100 intact mosquitoes with buffer occupy a volume that is greater than a standard 1.5 ml plastic centrifuge tube. Use of these tubes with microcentrifuges is important in keeping the assay simple and inexpensive. In contrast, hundreds of mosquito heads can be tested in a single tube. Another important consideration is that testing only the heads of mosquitoes imparts stage-specificity to the assay, since, with rare exceptions, only L3 larvae are found in the heads of mosquitoes. The mosquito PCR assay will be field tested in French Polynesia during the coming year.

The use of PCR to detect microfilariae in blood has also been a difficult problem, because blood also contains substances that inhibit the reaction. We have devised a rapid technique for processing blood samples for PCR that effectively removes these inhibitors and requires only three simple procedures. A 50-100 ul blood sample is digested with Proteinase K, extracted with phenol and chloroform, and dialyzed against a Tris/EDTA buffer. A few microliters of the dialysate is then added to the PCR reaction. 10 to 20% of the PCR reaction is loaded on an agarose gel and stained with ethidium bromide. Using this technique, a single microfilaria in 50ul of blood is detected in both the *Hha* I PCR system (*Brugia*) and the *Ssp* I system (*Wuchereria*). The intensity of the band in the gels indicates that a single microfilaria should be easily detected in 100 ul or more of blood. Experiments to demonstrate this are currently underway.

A field study using the *Brugia Hha* I PCR assay on human blood samples collected in Indonesia has recently been completed. In this study, the nuclepore filtration/microscopy method was used as the "gold" standard for evaluating the PCR assay. The PCR assay and the filtration/microscopy methods were in 100% agreement on all 64 of the microfilariae(+) samples and all 30 of the microfilariae(-) non-endemic normal samples. Of the 30 endemic microfilariae(-) samples (negative by filtration/microscopy), four were positive by PCR. These four blood samples were retested several times with the same results. These data indicate that the PCR assay is more sensitive than the filtration/microscopy method. The four microfilariae(-)/PCR(+) samples may represent cryptic infections or infections with very low numbers of microfilariae missed by the filtration/microscopy method. The possibility that circulating or "free" DNA was detected in these samples should not be overlooked.

The *Hha* I and *Ssp* I PCR amplification systems have also been used to detect circulating or "free" DNA in blood or serum. In reconstruction experiments, these PCR assays can be used to detect less than 0.5 pg of DNA added to 50 ul of human blood or serum. The *Ssp* I PCR amplification system has been used to detect "free" DNA in blood and serum samples from *Wuchereria* infected individuals on Mauke in the Cook Islands (research done in collaboration with Dr. James McCarthy and Dr. Eric Ottesen of the National Institutes of Health). The *Hha* I PCR amplification system has been used to detect "free" DNA in the blood of *Brugia* infected humans and jirds (research done in collaboration with Dr. T. Supali and Dr. F. Partono of the University of Indonesia).

Although use of the PCR reaction for screening mosquito and human blood samples has been criticized due to its cost, the PCR test may be less

expensive for testing vectors because they can be screened in pools rather than individually as with a labeled DNA probe hybridization system. Additionally, our PCR-based detection systems do not require a labeled DNA probe, dot blots, hybridization, washes, X-ray film, dark rooms or expensive detection reagents. Thus, the use of PCR is not only much faster than hybridization with a DNA probe, it is less expensive. As with DNA probe hybridization systems, PCR is best carried out in a centralized laboratory with good quality control.

We have initiated experiments to simplify the transfer of PCR technology to endemic regions. These experiments include freeze-drying PCR tubes containing all of the components of a PCR reaction except the template to be tested. The sample to be tested is simply added (with water) to the tube and placed in the thermal cycler. We are also testing the use of inexpensive, low-maintenance thermal cyclers. Our initial results in these studies are very encouraging.

3. Onchocerciasis Reports

3.1 Thomas Unnasch--DNA Probes and a PCR Amplification System for the Detection of *Onchocerca volvulus*

At the 1989 DNA Probes meeting in Jakarta, Indonesia, the development of strain-specific DNA probes was reported based on a highly repeated DNA element (O-150) cloned from *Onchocerca volvulus*. These probes could differentiate the pathogenic savannah strain of *O. volvulus* (associated with severe ocular pathology) from the less-pathogenic forest strain. Primers based on the O-150 repeat sequence were used to PCR amplify samples collected from individuals in three types of villages in the Onchocerciasis Control Program (OCP): savannah, forest and intermediate. The intermediate villages were located in areas of Sierra Leone where the rain forest had been cut down and is now converting to a savannah ecosystem. In these villages, there is an association between the community microfilaria load and ocular disease, but not as strong as the association in the savannah villages.

Following PCR, an aliquot of the reaction is run on an agarose gel, Southern transferred, and then hybridized with non-radioactively labeled strain-specific plasmid probes. These probes are pFS-1 (the forest-specific probe) and pSS-1BT (the savannah-specific probe). These probes, when labeled non-radioactively with digoxigenin, are sensitive enough to detect DNA from individual microfilariae and individual L3 larvae.

This study was designed to test the efficacy of the PCR/hybridization assay in relating the strain of the parasite to the epidemiological disease pattern. 17 villages in the OCP were selected for the study. Following PCR, the Southern blots were first hybridized with the forest-specific probe pFS-1. If no hybridization was seen, they were then hybridized with the savannah-specific

probe pSS-1BT. The results of the study showed a striking concordance between the strain (as identified by DNA hybridization) and the disease status of the individual. Only five isolates were misclassified and these all came from villages at the interface of the forest and savannah. Overall, the probes showed 93% sensitivity and specificity in this study.

The study also demonstrated that villages of the intermediate type in Sierra Leone had both forest and savannah strains of the parasite. One of the major vectors in Sierra Leone is thought to transmit both the forest and savannah strains of the parasite.

The need for accurate measurement of the annual transmission potential (ATP) in the OCP is complicated by the fact that *Onchocerca ochengi* L3 larvae are difficult to differentiate from *O. volvulus* L3 larvae. In order to develop a DNA probe that would differentiate these two species, several hundred of their O-150 repeats were cloned and sequenced. Based on these data, an *O. volvulus* specific oligonucleotide probe (Ovs-2) and an *O. ochengi* specific oligonucleotide probe (Och) were developed. These new probes recently proved valuable in a study done on infected flies collected in Mali and northern Cote d'Ivoire. These flies were collected from a region that had been under vector control for 15 years. When no further transmission of *O. volvulus* was observed, spraying was stopped in 1990. One year later, many infected flies were collected and spraying was resumed. For this study, 700 flies were collected and dissected and 13 were found to contain *Onchocerca* L3 larvae that were indistinguishable from *O. volvulus*. These 13 flies were PCR amplified using the O-150 primers. The O-150 repeat was successfully amplified from 11 of these flies. None of these PCR products hybridized with the *O. volvulus* specific probe (Ovs-2) while 10 of the 11 samples hybridized with the *O. ochengi* probe (Och). Based on these data, spraying in the region was again halted. The annual savings to the OCP is estimated to be \$35,000 to \$40,000 per year.

3.2 Laurent Toe--Use of Species-Specific and Strain-Specific DNA Probes for *Onchocerca volvulus* in the OCP

Note: Dr. Toe is in charge of the DNA probes field laboratory that was established in the OCP in Cote d'Ivoire in 1992.

The objectives for our work using the *Onchocerca* DNA probes in the OCP are as follows: 1) to define the distribution of blinding Onchocerciasis in the OCP area, 2) to define the annual transmission potential (ATP) of blinding onchocerciasis at OCP catch points both inside and outside the OCP area, and 3) to assess the vectorial role of *Simulium damnosum* species for *O. volvulus* and other species of *Onchocerca*.

Individual L3 larvae are dissected from black flies, PCR amplified using the O-150 primers, and then hybridized with the species-specific oligonucleotide probes (Ovs-2 and Och) and the strain-specific plasmid probes (pFS-1 and pSS-1BT) as described in Tom Unnasch's report above. Data are now being collected

from about 100 mosquito catch points covering 11 countries in the OCP area to study the relative distribution of *O. volvulus* and *O. ochengi*. Using the Ovs-2 probe, *O. volvulus* has been identified from all regions of the OCP area. The Och probe has identified *O. ochengi* in large numbers from Mali, but samples have also been identified from Sierra Leone, Benin, Cote d'Ivoire, and elsewhere in the OCP area. The strain-specific probes are being used to study the distribution of the savannah and forest strains of *O. volvulus*. The savannah strain is found mostly in the savannah areas in the north, but with continued forest destruction it is also found near the coast in Cote d'Ivoire and Togo. The forest strain is found only in the forest regions of the south.

A few isolates from Mali are a cause of some concern. These isolates PCR amplify with the O-150 primers but do not hybridize with either the *O. volvulus* or *O. ochengi* species-specific oligonucleotide probes. The species identity of these isolates is unknown.

3.3 Stefanie E.O. Meredith--A PCR System for Detecting *Onchocerca volvulus* in Black Flies and Blood Samples

The use of PCR to amplify *O. volvulus* repeat DNA obtained directly from *Simulium* vectors treated by Proteinase K digestion and boiling did not work (this simple method works for the detection of *Leishmania* in *Phlebotomus* vectors). A strong inhibitor in the black fly (perhaps a component of the cuticle) must be removed and the remaining inhibiting substances must be diluted in order for the assay to successfully amplify *O. volvulus* repeat DNA. The successful protocol involves incubating the entire fly in dithiothreitol (DTT), Proteinase K and Triton X-100 to break up the L3 larvae. The digested material is then centrifuged to remove the cuticle and other debris. The sample must then be diluted 1:20 up to 1:100 prior to PCR. An aliquot of the PCR reaction is run on an agarose gel, Southern transferred, and hybridized with species or strain-specific *Onchocerca* oligonucleotide probes.

This system was used to identify strains of *Onchocerca* from *Simulium* vectors collected in Cote d'Ivoire in 1987. These flies were all of the same species and were from the same region. They were fed on three volunteers from three different regions in the OCP area. The flies were then killed at various time points to ensure collection of flies with microfilariae, sausage stage, L2 and L3 larvae. The flies were dried and then stored with silica gel for five years. Following the above protocol to break the vector and the L3 larvae, three different oligonucleotides were used as hybridization probes. One probe was species-specific for *O. volvulus*, another was a savannah-specific probe, and the third was an *O. ochengi*-specific probe. All stages of the parasite were detected. The flies fed on the volunteer from a savannah region of Mali hybridized with the *O. volvulus* specific probe but not the savannah probe. This finding agrees with Dr. Laurent Toe's observation of samples from this region of Mali that do not hybridize with their *O. volvulus* savannah specific probe. One fly fed on

another volunteer did not hybridize with any of the three probes. Repeats from this mystery sample are now being sequenced.

The PCR system was also used to examine the blood of European patients returning from Africa with *O. volvulus* infections. Ten of these Europeans were negative for microfilariae on skin snip examination but were positive for *Onchocerca* antigens and had a positive Mazzotti reaction indicating they were infected. Five ml of whole blood was taken from these individuals in EDTA vacutainer tubes. The samples were digested with Proteinase K, extracted with phenol and chloroform, and then ethanol precipitated. The pellets were resuspended in TE and a small sample was taken for PCR amplification. All of these clinically proven *Onchocerca* positive individuals were also PCR positive even though they had no microfilariae either in skin snips or in the blood.

Endemic samples were recently collected from Cote d'Ivoire and processed in the same way. All samples collected from a region with no onchocerciasis were PCR negative. For individuals from the onchocerciasis region, no correlation was seen between the strength of the PCR signal and the microfilariae levels from skin snips. The strongest signal in the first 31 samples tested, came from a microfilariae negative woman with leopard skin.

Many difficulties remain with this method. There are problems of inhibition of the PCR reaction by factors in the blood. The best solution to this problem so far, has been to check the amount of human DNA by spectrophotometric readings and then dilute the sample if the amount of human DNA is too great. Despite these difficulties, this method shows great promise for detecting *Onchocerca* infections in individuals who are microfilariae negative.

3.4 Peter Zimmerman--A PCR Based Method for Detecting *Onchocerca* DNA in Skin Snips

A method has been developed for PCR testing skin snip samples for the presence of *Onchocerca volvulus* DNA. The purpose was to develop a method that could be used to rapidly assess the epidemiology of the disease in an endemic region. Two important questions to be addressed were: 1) is the PCR technique more sensitive than microscopic examination of skin snip fluid? and 2) is there any correlation between the number of microfilariae observed and the PCR signal?

For this method, the skin snips were incubated for 24 hours in saline and examined microscopically. The samples were then transferred to 1.5 ml plastic centrifuge tubes containing 500 ul of 100 mM EDTA. The samples were then frozen until the procedure could be completed. Later, the samples were thawed and digested in Proteinase K and SDS for one hour. Dithiothreitol (DTT) was then added to a final concentration of 20 mM and the samples were boiled for 30 minutes. The samples were then rapidly freeze/thawed three times and then ethanol precipitated. The pellets were resuspended in 25 ul of TE buffer. A few microliters were then PCR amplified using the *O. volvulus* O-150 primers. 25% of

the PCR product was then run on a 2% agarose gel and stained with ethidium bromide.

The results on the 72 samples were that 48 were positive upon microscopic examination of the skin snips, while 56 were positive by PCR. Conversely, 24 of the samples were negative by microscopic examination, while only 16 were negative by PCR. The eight PCR positive samples that were microscopy negative indicate that the PCR assay is much more sensitive than the standard skin snip examination. Upon comparison of the numbers of microfilariae counted by microscopic examination and quantitation of PCR products following ethidium bromide staining, it was found that these two methods could not be reliably correlated. Thus, the PCR-based assay should not be used to determine intensity of individual infections. This technique may prove useful in addressing epidemiological questions such as the following. 1) Can the assay be used to assess the kinetics of microfilaria clearance and reappearance following ivermectin treatment? 2) Can this assay be used to determine if children are being exposed to *O. volvulus* by detection of pre-adult larval stages?

4. Vector Reports

4.1 James B. Lok--A Review of Vector Biology and Filarial Parasite Transmission

A summary of the sequence of development for both *O. volvulus* and the lymphatic filariae was presented. A relevant point for using DNA probes to screen vectors is that an already infected host-seeking black fly or mosquito does not carry any blood from the previous blood meal. All of the filariae within the vector will be in the late L1, L2 or L3 stages of development. Thus, if human bait is used to trap vectors, only L3, L2 or L1 larvae that have escaped the gut will be detected by an appropriate DNA probe.

A review of the quantitative aspects of infection was presented. Attrition rates within the vector may be high due to gut dissemination barriers. One such barrier is seen in *Simulium ochraceum* where significant numbers of *O. volvulus* microfilariae are shredded by sharp spines of the cibarial armature as they traverse the foregut. Those microfilariae that survive this process are very likely to complete the developmental steps to the L3 stage. This implies that the development of *O. volvulus* in the thoracic muscles of *S. ochraceum* is a very efficient process. In contrast, members of the *S. damnosum* complex lack the highly developed cibarial armature seen in *S. ochraceum*. Here the non-cellular peritrophic membrane is the most important modulating factor. In these species, about one-half of the *O. volvulus* microfilariae make it out of the peritrophic membrane that surrounds the blood meal. As was the case with *S. ochraceum*, those microfilariae that surmount this barrier are very likely to develop to the L3 stage. An example in the lymphatic filariae that has been studied is the

development of *W. bancrofti* in species of the *Anopheles gambiae* complex. These vectors have a well-developed cibarial armature that represents a significant barrier to the microfilariae. Here, only about one-half of the filariae that make it to the thoracic flight muscles survive. Thus, *A. gambiae* species are highly inefficient vectors with both dissemination and developmental barriers. In contrast, *Culex quinquefasciatus* is a more efficient vector with most microfilariae successfully surviving to the L3 stage. However, even in this species, genetic factors that bring about abortive development of larvae within the thoracic musculature have been identified in certain populations. The mode of expression of these factors is presently unknown.

Data were presented on the prevalence of infected and infective vectors both before and after mass chemotherapy. Data from an *O. volvulus* focus in Guatemala showed a significant decrease two years after mass chemotherapy in the number of L3 larvae per 1000 parous flies dissected (from a mean of 20 per 1000 to a mean of 3 per 1000). Similar results were seen in the *Aedes polynesiensis* vectors of *W. bancrofti* in Samoa. Here the fraction of infected vectors decreased after mass chemotherapy from 2.95% (12 infective out of 407 dissected) to 0.07% (3 infective out of 4290 dissected). These data illustrate how highly efficient vectors such as members of the *Ae. polynesiensis* complex can bring about resumption of *W. bancrofti* transmission even with the low microfilaremias prevailing after mass chemotherapy, and they underscore the need for highly sensitive probes to detect microfilariae in peripheral blood. It is also clear from these findings that effective monitoring of control programs requires the screening of very large numbers of vectors.

The use of DNA probes to screen only the heads of vectors would impart stage-specificity on the assay, since only L3 larvae are found in the head. A published method to isolate and purify the heads from thousands of mosquitoes was described. This method involves freezing the vectors at -70°C and then shaking them rapidly to break the heads away from the rest of the body. The body parts are then separated through standard sieves and the purified heads are collected by aspiration. This method is usually used on 100,000 insects per preparation. Thus, many thousands of vector heads could rapidly be prepared by this method for DNA probe screening.

4.2 Thomas R. Burkot--Field Considerations in the Development of Filarial Detection Methods for Vectors

Filarial worm detection in vectors will be most useful in the field for monitoring transmission and for incriminating new vectors. Filarial worm detection methods for field or epidemiological use must be compatible with commonly used vector collection and storage methods in developing countries. In addition, they must be capable of processing the numbers of vectors to allow statistically significant results to be obtained. Sensitivity of such detection systems must be one worm. Specificity must be at least to the species level and ideally to the infectious stage. While comparative infection rates using non-

stage-specific probes will allow estimates of transmission using an assumption that a certain proportion of infected vectors will be infectious, such an approach will underestimate the efficacies of interventions which affect vector survivorship and will prohibit attempts to measure the real transmission rates in an area. Conversely, assaying only the heads of vectors will yield underestimates of the proportion of infectious vectors. The development of stage-specific probes would enable estimates of vector survivorship through comparisons of infectious to infected vectors.

Interventions directed against the helminth or its vector must use the village as the basic sample unit. The large fluctuations in transmission of vector-borne diseases in villages prohibits the use of a village as its own control. In addition, the use of nonparametric tests of significance requires a minimum of 6-8 pairs of villages in evaluations of an intervention. An analysis of L3 infection rates in Tanzania gave an estimate of more than 40,000 mosquitoes to be assayed to obtain significant results if a study of DEC efficacy had been carried out for an additional year. Collection and storage of such large numbers of vectors in the tropics is most practical if they can be dried and held at laboratory temperature.

As the proportion of infectious flies diminishes in the population, the proportion of false to true positives in an assay system will increase. One way to minimize the false positive rate is by assaying pools of vectors. Therefore, the ideal field test would be of sufficient sensitivity to process pools of vectors. If the proportion of positive pools is held below 5%, there is a greater than 95% probability that a positive pool resulted from a single infected vector.

An examination of human pathogen detection systems for malaria and Lyme disease shows PCR to be the most sensitive method followed by antigen detection systems and then DNA hybridization. Further attempts at developing stage-specific antigen capture assays are therefore warranted. Questions as to epidemiologically relevant sensitivity are raised as well as the interpretation of positive results. Does a positive result indicate an infectious fly, an infected fly, or merely a fly exposed to the pathogen?

The inoculation rate consists of estimates of both the proportion of infectious flies and the rate of human-vector contact. It is argued that the magnitude of error associated with estimates of the human-vector contact is greater than the error of the proportion of infectious vectors and efforts need to be directed towards this area of research. Efforts should also be made towards measuring the transmission rate to humans through the use of stage-specific antigens in serological tests.

4.3 Willy Piessens--Applications of DNA Probes to Infected Vectors in China

This report is designed to address the following questions: 1) In China, what is the sensitivity required for a DNA probe to be useful in screening vectors? 2) Can a non-stage-specific DNA probe be used to screen intact mosquitoes to obtain an estimate of the infectivity rate? 3) Can the heads of vectors be screened to obtain an estimate of the infectivity rate? 4) In China, is an

L3-specific DNA probe needed? In collaboration with Dr. Zheng in China, these questions are currently being addressed.

1) What is the sensitivity required for a DNA probe? In surveys in villages in China, infectivity rates were often found to be very low. In one village, two L3 larvae were found in 100,000 dissected mosquitoes. At this level of infectivity, no further transmission is observed in this village. In China, they would like to be able to reliably detect one L3 in 100,000 mosquitoes using a method more rapid than dissection.

2) Can a non-stage-specific DNA probe be used to screen intact mosquitoes to estimate the infectivity rate? In China, a good correlation was observed between the microfilaria rate in humans and the infection rate in mosquitoes. However, the infection and infectivity rates in mosquitoes varied greatly over a period of one year. For the infection rate, the values ranged from 0% in the winter to about 25% in the summer. The infectivity rate ranged from 0% in the winter to 2.3% in the summer. Furthermore, the ratio between the infection rate and the infectivity rate was not constant over time. These ratios vary from 0.2 to 0.6 for *Brugia* and 0.1 to 0.2 for *Wuchereria*. Because the ratio is not constant over time, it would be difficult to extrapolate from infection rates (determined by using non-stage-specific DNA probes on intact mosquitoes) to infectivity rates.

3) Can the heads of vectors be screened to obtain an estimate of the infectivity rate? In a study done by C.P Ramachandran, it was found that L3 larvae were distributed throughout the bodies of laboratory reared mosquitoes. About half were found in the head and half elsewhere in the body. Thus by examining heads only, DNA probes would show excellent specificity for L3 larvae, but would show only about 50% sensitivity. If this 50% were constant, the number of L3 larvae in the body could easily be extrapolated from the number of L3 larvae in the head. Unfortunately, it was found that the ratio of L3 larvae in the head to L3 larvae in the body changes over time. Early in development of the L3 larvae, many are found in the thorax. Later more L3 larvae are found in the head. An experiment done in 1945 demonstrated that L3 larvae migrate throughout the mosquito and that this migration appeared to be random. Therefore, probing only the heads of mosquitoes may not be a useful way for estimating infectivity rates.

4) Is an L3-specific DNA probe needed in China? In China, a stage-specific (i.e. L3-specific) DNA probe is not necessary, because the government does not want to see any filariae of any stage in the mosquitoes. Filariae of any stage would indicate at least the potential for transmission.

4.4 Julian Crampton--DNA Probes for the Species Identification of Vectors of Filariasis

DNA probes for vectors of filariae can be used to identify sibling species within a species complex that are difficult or impossible to distinguish morphologically. These probes should be reliable, simple to use and inexpensive. The strategy we have used to develop these DNA probes is to use "shotgun" cloning to make genomic libraries and then screen these libraries for highly repeated DNA sequences.

An example of the development of these probes are the ones developed to differentiate the six species of the *Anopheles gambiae* complex. The original probe that was developed hybridized to all six of the species. Further cloning and sequencing of repeats from these species has led to the development of species-specific oligonucleotide probes. Most of the repeats used are tandemly repeated and located near the centromeres and telomeres of the chromosomes. There are now probes available for all six of the species in this complex.

Another example is the development (with Rory Post) of DNA probes for the *Simulium damnosum* complex. Here, DNA probes have been developed that identify flies down to the sub-complex level, but not the species level.

The use of such probes in the identification of insect vectors has been refined to make the assays more practical for field use. In black flies, for example, DNA isolation is not necessary. Flies are simply placed between two sheets of nitrocellulose membrane and squashed. This produces duplicate "squash blots" that can be hybridized simultaneously with two different DNA probes. To simplify the hybridization procedures, a hybridization solution has been developed that consists only of salt, SDS and non-fat dry milk. All of the hybridizations can be done in this solution at 25°C so that heating water baths are not required. Many non-radioactive labeling and detection systems were evaluated for sensitivity, low background, and low cost. Here the E-Link Plus system from ICI proved to be the most sensitive and the least expensive. These probes were found to be stable at 4°C for at least six months. One technician could screen about 300 flies per day at a cost of about \$0.02 to \$0.04 per fly. Detection of the hybridized probe could be done using chemiluminescent or colorimetric reagents. This method was also found to be effective for mosquito squash blots (including adult mosquitoes and larvae).

In summary, the DNA probes and assays for screening vectors have the following properties: 1) the probes are non-radioactive, 2) the assay uses simple, inexpensive solutions, 3) the hybridizations are done at room temperature, 4) the probes are stable for at least six months in the refrigerator, 5) the assay is inexpensive, 6) the probes can be used to screen adults or larval stages, 7) 300 insects can be screened per technician per day, 8) the assay works on dried insects or on insects preserved in alcohol, 9) the assay requires very little equipment, and 10) the squash blot filters can be stripped and reprobbed.

5. Priorities for Research

Following the reports on the use of DNA probes and PCR to detect filariae in vectors and blood, the participants divided into groups to discuss four major areas of concern. These small groups reported their discussions and recommendations to all of the participants. The entire group then discussed these recommendations for research. The four areas of discussion were as follows. 1) What is the sensitivity and specificity required for DNA probes or PCR in vector studies? 2) Are stage-specific (i.e. L3-specific) DNA probes necessary? 3) What are the technical problems that need to be addressed for using DNA probes and PCR to detect filariae in vectors and blood? 4) What field questions require these DNA probes and/or PCR and are any new probes needed?

5.1 What is the sensitivity and specificity required for DNA probes or PCR systems for vector studies?

DNA probes and PCR systems for lymphatic filarial parasites and for *Onchocerca* are available which have the required sensitivity, namely the ability to detect a single L3 in a vector. The probes for lymphatic filariae are species-specific, while both species-specific and strain-specific probes exist for *Onchocerca volvulus*.

Questions remain with regard to whether abortive filarial infections in secondary or non-vector species will render false positives in DNA based detection systems. Previous research has identified several mechanisms of parasite killing or arrest in vectors including gut level barriers, cellular or humoral defenses outside of the gut, and genetically based factors operating at the tissue site of parasite development. Research is needed to determine whether DNA from parasites arrested or killed by these various mechanisms can persist in the vector and act as a template for recognition by DNA probes or PCR primers. This is an especially important consideration when DNA probes or PCR are being used to incriminate a new vector.

DNA probes and PCR will be used for both vector incrimination and monitoring control programs. Vector incrimination studies will necessarily involve the use of DNA probes or PCR to identify parasites in concert with vector dissections. Dissection will yield information on the location of L3 larvae within the vector, while DNA probes or PCR will be used to determine the species identity of the L3 larvae.

Dissections of field collected vectors should focus on determining the infection rate as well as the infectivity rate. Assessment of the site of infection with respect to major body parts of the vector should also be included. Pilot studies in the laboratory may be necessary to elucidate details of parasite migration within the bodies of vectors. This latter information will be essential in determining the efficacy of measuring infection in whole vectors versus isolated heads of vectors.

Currently available probes lack stage-specificity. Given this fact, incrimination of vectors based on DNA probe recognition must be corroborated by laboratory studies demonstrating that parasite development in that species proceeds to the third larval stage and that L3 larvae are transmitted by bite. That is, it must be demonstrated that the vector is truly competent to transmit the parasite.

The sensitivity of PCR systems for determining levels of infectivity should be compared between pools of intact vectors and pools of isolated heads. Assaying individual heads will be necessary in the initial incrimination of vectors. In addition, assays of pooled heads rather than intact vectors may be necessary in order to maintain sample volumes within practical limits.

Whether it is most appropriate to probe intact vectors or isolated heads in screening a vector population may depend on the level of infection in that vector population. If the level of infection is extremely low (for example in a control program region where only a few vectors in 100,000 are infected), it may be most appropriate to probe pools of intact vectors to determine if any of the vectors are infected. On the other hand, if the level of infection is on the order of one in 1000, it may be best to probe pools of isolated heads to get an estimate of infectivity.

For monitoring a control program, the most appropriate method for screening vectors may depend on the method of control being used. If the control program is directed against the vector, then knowing the level of infectivity is crucial. This is because vectors that are merely infected may not live long enough to produce L3 larvae. Using PCR to amplify all of the stages from intact vectors would amplify L1 and L2 larvae and might lead to an interpretation that the control was not effective when in fact just the opposite might be true. Under these circumstances use of either an L3-specific probe or use of PCR to screen only isolated heads of vectors would be the most appropriate screening strategy. If the control program is directed against microfilariae in the definitive host, then use of a non-stage-specific probe or PCR assay on intact vectors would be reasonable.

Monitoring infection in vector populations (particularly during a control program) will require, by nature of the diminishing infection rates, assaying pools of vectors to determine infection rates. In order to accurately determine the infection rate, the proportion of positive pools must be kept below 5% to ensure a 95+% probability that a positive pool is the result of a single infected vector regardless of pool size. The development of an index (such as the minimum infection rate index used by arbovirus epidemiologists) would be based on the assumption that a positive pool is due to a single positive vector within that pool. Further laboratory research will be required to assess the effect of increasing pool size on the ability of the PCR systems to detect a single filarial worm.

Research Needs

- 1) The sensitivity and reliability of PCR-based systems for detecting a single parasite in pools of vectors must be increased. Sensitivity of the PCR assay must be compared between pools of intact vectors and pools of isolated heads.
- 2) DNA probes and PCR systems should be utilized for vector incrimination. In such studies, the DNA methods should be used to unambiguously speciate the parasite in the vector. Dissection should be used to determine the infection and infectivity rates in the putative vector. Classical parasitological techniques need to be used to verify that the vector is competent for transmission.
- 3) The location and migration of L3 larvae within each vector species should be studied over time. Such data will aid in the interpretation of DNA studies based on screening only the heads of vectors.
- 4) Can DNA from parasites arrested or killed persist in a vector and be detected by PCR? This needs to be determined before DNA PCR methods can be used to incriminate new vectors. Non-competent vectors may take up a parasite and kill it or block its development. DNA would likely be released and may be detected by PCR. This is not likely to be a problem if human bait is used to collect the vectors, because infected vectors will seek human bait only after they have passed their first blood meal. If a parasite is killed within the gut of the vector, most if not all of its DNA would likely be passed along with the first blood meal. On the other hand, infrequent instances of abortive larval development within tissues of secondary vector species might result in liberation of parasite DNA which could yield false positive results in a PCR-based assay.

5.2 Are stage-specific (i.e. L3-specific) DNA probes necessary?

The answer to this question is probably no, especially since developing such probes would be extremely difficult. In many situations, decisions about the initiation or effectiveness of control efforts can be made solely on information indicating that parasites are still in the environment. In such cases, stage-specific probes would not be necessary.

The major reason for wanting to know if L3-stage parasites are present in vectors is to be able to calculate infectivity indices. If probes detecting parasites are not stage-specific, uncertainty is introduced because there is not a 1:1 relationship between early-stage larvae (i.e. microfilariae in blood meals, etc.) and infective-stage larvae. Experimental studies for *O. volvulus*, *B. malayi*, and *W. bancrofti* all indicate that once larvae have developed to the point that they enter the thoracic flight muscles (sausage-stage or L2 larvae), they essentially all develop into L3 larvae. Thus, the major source of the 'uncertainty' or inaccuracy in estimating L3 numbers from probes that detect all larval stages is that larvae in recently acquired blood meals will give positive signals, yet most will not

develop into L3 larvae. The percentage that will not develop into L3 larvae is not predictable particularly during interventions affecting vector survivorship (e.g. permethrin impregnated bed nets). A second cause of the uncertainty or inaccuracy in estimating numbers of L3 larvae is the fact that secondary vectors (i.e. relatively inefficient ones) may not have the same 1:1 larval development ratio as do the more efficient primary vectors. That is, sausage-stage or L2 larvae that make it to the flight muscles might not all develop into L3 larvae. The ratio will be less than 1:1 but will probably be fairly constant for a given vector species.

There are two practical ways to avoid the first and more important uncertainty in estimating infective larvae from non-stage-specific DNA probes. First, one should sample only those vectors without recent blood meals (in which all the microfilariae and early larvae are found). Such discriminatory sampling can be done by using human bait catches for both flies and mosquitoes, since only nulliparous and parous vectors would be attracted to the bait. (Parous females generally do not return for a second blood meal until the first blood meal has been digested and passed). Other vector trapping techniques (light traps, CO₂, indoor resting, etc.) would catch vectors containing recent blood meals, the source of most of the uncertainty resulting from use of the non-stage-specific probes. Second, one should examine only those parasites located in the heads of the vectors, since these will all be L3 larvae. Though such a technique would underestimate the number of L3 larvae by about 50%, specificity is very high and increasing the number of vector heads examined would improve the sensitivity. An efficient technique for the collection of vector heads has already been described in Dr. James B. Lok's report above.

The lesser cause of uncertainty in using non-stage-specific probes on intact vectors is from attrition of developing larvae. This occurs in less efficient secondary vectors where the ratio of developing (early flight muscle) larvae to infective L3 larvae is less than the nearly 1:1 ratio found in the optimal vector. If one cared to minimize this cause of uncertainty, relative vector competence could be determined for each vector species, and if the proportion of different vectors biting in a locale where transmission was being assessed was determined, then corrective calculations could be applied to data obtained with non-stage-specific probes. Again, this problem could be avoided by using a non-stage-specific probe to screen only the heads of vectors.

Research Needs:

1) Development of new transmission indices based on the type of data that comes from the use of non-L3 specific probes. This work would involve direct measurements in the same environment of transmission indices by the standard and probe-based techniques. The mathematical modeling required to develop indices based on PCR/probe based methods may be quite complex, since the relationship between the infection and infectivity rates varies with each vector species and also varies with the season. Other environmental factors may also be important. If such models can be successfully developed, then data obtained

from non-stage-specific PCR/probe methods may be translated into the annual transmission potential (ATP) that entomologists are accustomed to using.

- 2) Development of techniques to quantitate parasite detection from PCR positive signals (for example, generation of estimates of the number of larvae in various size 'pools' of vectors being sampled).
- 3) Development of techniques to purify vector heads for analysis or to enrich for L3 larvae collected from vectors.

5.3 What are the technical problems that need to be addressed for using DNA probes and PCR to detect filariae in vectors and blood?

Tremendous progress in the technical area has been made since the DNA Probes meeting in Jakarta in 1989. For example, at the Jakarta meeting, much discussion focused on how to break open L3 larvae to extract DNA. This is no longer a problem. The technical problems that now need to be addressed for using DNA probes and PCR to detect filariae in vectors are straightforward and will require further research.

Research Needs in Vectors

1) How sensitive can the PCR method be for detecting *Brugia* or *Wuchereria* in mosquitoes and for detecting *Onchocerca* in black flies? In order to maximize the sensitivity and reliability of the assay, the vector components that inhibit PCR must be eliminated. Alternatives to the current methods of organic extraction and silica should be investigated. Other materials such as ion exchange resins should be tested for their ability to eliminate the inhibitors. Other thermostable polymerases besides *Taq* polymerase and other amplification techniques should be tested in efforts to increase the sensitivity and reliability of the assay. The sensitivity and reliability of the assay should be optimized for both intact vectors and isolated heads, so that both are available to the scientific community.

2) Methods for long term storage of infected vectors should be tested in field studies. S.E.O. Meredith reported success in PCR amplifying *Onchocerca* from black flies that had been dried and stored with silica gel for five years. This same method should be tested for mosquito vectors carrying lymphatic filariae. S.A. Williams reported a method for storing infected mosquitoes in 100mM EDTA. This method should be compared to the drying method to determine which is the simplest and most reliable for preserving thousands of intact vectors for PCR.

3) The PCR method for screening vectors should be tested in the field as a method for evaluating chemotherapy programs.

4) Other technologies not related to DNA should be investigated for detecting infected or infective mosquitoes. For example, a histochemical stain that bound only to L3 larvae could be used to easily screen thousands of

mosquitoes rapidly. Unfortunately, such a method is unlikely to be species-specific. In regions where a vector commonly carries only one species of filaria, such a method would be very useful.

Research Needs in Blood and Other Non-Vector Samples

1) Do the PCR results presented at this meeting by S.A. Williams, S. Dissanayake, and S.E.O. Meredith demonstrate that "free" DNA is being detected in the blood of infected patients? Further research with larger numbers of samples and all appropriate controls must be done to demonstrate the validity of this assertion.

2) What is the best method for preserving blood samples for the detection of "free" DNA? Is EDTA sufficient or do the samples also need to be frozen (and at what temperature)? What is the best method for processing the blood samples to eliminate inhibitors and optimize the sensitivity and reliability of the PCR reaction? What is the best fraction of the blood to screen for circulating DNA (whole blood, serum, or plasma)?

3) What is the relationship, if any, between the amount of DNA produced by PCR amplification of "free" DNA and the microfilaria level in the host?

4) Can "free" DNA be detected in the urine, tears or saliva of infected patients? How would these samples be collected, stored and processed for PCR?

5) Is the positive PCR signal in skin snip negative *Onchocerca* patients (reported by P. Zimmerman) due to "free" DNA in the snips or due to non-motile microfilariae?

5.4 What field questions require these DNA probes and/or PCR and are any new probes needed?

Field Applications and Research Needs

The field applications that the DNA probes and PCR systems should address in vectors are as follows: 1) Use of PCR on pools of vectors to measure the minimum infection potential (rather than the annual transmission potential). This value assumes that each positive pool is due to a single infected vector. For this to be valid, the size of the pools must be adjusted so that the number of positive pools is below 5%. As discussed in previous sections, it may be possible to relate the minimum infection potential measured by PCR to the annual transmission potential using a mathematical model. 2) Use of PCR on pools of vectors to measure the impact of control measures on the minimum infection potential (which, again, may be related to the annual transmission potential). 3) Use of DNA probes and/or PCR to measure recrudescence of vectors in control program regions. 4) Use of DNA probes to incriminate new vectors (theoretically, different DNA probes could be used on the same vector to speciate the vector and the parasite and to check the source of a blood meal).

Two non-vector field applications were discussed. 1) The use of PCR and/or DNA probes to monitor the effect of a chemotherapy program on the community parasite burden. 2) The use of DNA probes and/or PCR assays to detect microfilariae or "free" DNA in human blood or in skin snips (*O. volvulus*).

Are new DNA probes needed? Excellent species-specific probes and PCR amplification systems are available for *B. malayi*, *W. bancrofti* and *O. volvulus*. It is unlikely that any new probes will be needed. The species-specific probes for *O. volvulus* are specific in the Onchocerciasis Control Program region in Africa, but prior to initiating a new screening program in a previously untested region, the probes should be validated in a small pilot study. Currently, some excellent probes are available for speciating vectors (see J. Crampton's report above). New probes for *Simulium* need to be developed since many species are very difficult to differentiate. Such probes will be very useful for studies on the transmission of *Onchocerca*.

Several operational issues were also discussed. 1) DNA probes and PCR assays are best conducted in centralized laboratories where assays would be conducted by qualified personnel using standardized protocols with excellent quality control. The Onchocerciasis Control Program is an example of a successful model in an endemic area. 2) The assays should be simplified and made as inexpensive as possible. 3) The assays should be made as sensitive as necessary and as reliable as possible.

Finally, DNA probes have only been discussed at this meeting for the lymphatic filariae and *Onchocerca*. A DNA probe for *Loa loa* was developed by Amy Klion and Thomas Nutman. DNA probes do not yet exist for *Mansonella ozzardi* or *Mansonella streptocerca*. Since these species are of minimal health concern, developing DNA probes for these species should be a low priority.

5.5 Some Final Issues

Screening of vectors is non-invasive and easier to conduct than surveys of human populations. Parasitologists, morphologists, quantitative epidemiologists, entomologists and molecular biologists clearly must improve communication and work together to answer many of the important questions regarding the use of DNA probes and PCR to screen vectors. An effort must be made by scientists and funding agencies around the world to train young scientists from endemic regions in the new field of 'molecular entomology'. If as much progress can be made in the next three years as in the three years since the last DNA Probes meeting in Jakarta, then we will see many examples of successful field studies using DNA probes and PCR on vectors by 1996.

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