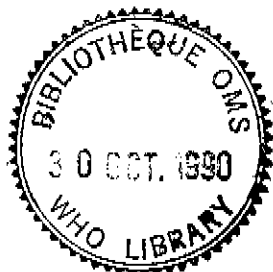




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UNDP/WORLD BANK/WHO SPECIAL PROGRAMME FOR
 RESEARCH AND TRAINING IN TROPICAL DISEASES



WORKSHOP ON DNA DIAGNOSTICS AND FILARIASIS

AND

SYMPOSIUM ON FILARIASIS AND ONCHOCERCIASIS

Jakarta, Indonesia, 18-20 December 1989

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1. INTRODUCTION

The Symposium on Filariasis and Onchocerciasis and The Workshop on DNA Diagnostics and Filariasis were held at the Hotel Indonesia, Jakarta, Indonesia. The Symposium and Workshop were sponsored by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and New England Biolabs, Inc. The purpose of the meeting was to review the progress made in the development of DNA probes for lymphatic filariasis and onchocerciasis and to study the possible application of these DNA probes in endemic regions. The Workshop on DNA Diagnostics and Filariasis was preceded by a one day Symposium on Filariasis and Onchocerciasis. The proceedings of this symposium are included as Appendix I.

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.

2. WORKSHOP ON DNA DIAGNOSTICS AND FILARIASIS - SUMMARY OF DISCUSSIONS

Dr. Larry A. McReynolds of New England Biolabs opened the workshop proceedings with a discussion summarizing the objectives of the meeting. The three primary goals of the meeting were: 1) to review the state-of-the-art of DNA diagnostic probes for the detection of filarial parasites. A discussion of the results of the coded dot blot filters sent to participants for hybridization with their DNA probes will be important in achieving this goal. 2) A second goal was to analyze the problems encountered in developing these diagnostic tools. Methods for increasing the specificity, sensitivity and field applicability of DNA probes will be discussed. Will DNA probes prove useful in replacing or supplementing standard techniques for detecting parasites in insect vectors and/or in the human host? It is clear that DNA probes may prove useful in some but not all field situations. Where are these DNA probes likely to prove most useful? 3) These questions must be considered in order to make recommendations to TDR and WHO on the development and use of DNA probes for filariasis. Recommendations should be made regarding the applicability of DNA probes to detect parasites in the vector and mammalian hosts. Recommendations should also be made on developing criteria for testing DNA probes and also on the development of new DNA probes and probe detection systems.

Considerations for the use of DNA probes in screening vector populations include a careful examination of the probe's sensitivity and specificity. In most areas, only a small fraction of vectors will actually be infected. This will be especially true in areas where parasite control programs are being monitored. This means that batch testing of hundreds or thousands of insects may be necessary. Such an approach will require DNA probes that are extremely sensitive. Problems in dealing with background hybridization due to insect parts such as wings and scales will also have to be dealt with. Because many vectors of human filarids can also carry animal filariae, the DNA probes used in screening vector populations must be extremely species-specific.

When considering the use of DNA probes to screen human host samples, sensitivity and species-specificity are also important. In terms of sensitivity, the issue must be raised as to whether DNA probes will be easier or more sensitive than standard methods in screening night blood or skin snips. The question of whether parasite DNA can be identified in the absence of microfilariae needs to be addressed. Specificity may be less of a problem in screening human samples than in screening vectors, but this issue must be discussed. In some cases, exquisite specificity may be useful; for example, in differentiating between blinding and non-blinding forms of Onchocerca volvulus.

Examples of the some of the possible uses of DNA probes are as follows. 1) DNA probes may prove useful in differentiating between the forest and savannah strains of Onchocerca. 2) DNA

probes may be used to monitor vectors in control areas such as the Onchocerciasis Control Programme. 3) DNA probes may be used to distinguish host range variants of a species, such as the anthropophilic and zoophilic types of B. malayi in Indonesia. 4) DNA probes may prove more sensitive than examination of night blood smears and may be able to detect occult infections. Could a DNA assay be used to screen day blood samples? 5) DNA probes may prove useful in studying transmission of the parasite following DEC or ivermectin treatment in community trials. In order to do this the probes must be extremely sensitive. 6) Finally, knowledge gained in the development of DNA probes may prove useful in the study of parasite evolution and taxonomy.

2.1. Onchocerca and Loa loa: Diagnosis

The first scientific session of the Workshop on DNA Diagnostics and Filariasis was chaired by Dr. Bruce M. Greene.

2.1.1. Diagnosis of Loa loa Infections--Dr. Thomas Nutman

Loa loa, the African eye worm, is a filarial parasite with a life cycle that is very similar to that of Onchocerca or the lymphatic filarial parasites. These parasites can migrate through the eye of the human host, but they generally do not cause permanent ocular damage. The vectors are tabanid flies of the genus Chrysops. Infected individuals in endemic areas tend to be microfilariae positive and asymptomatic or amicrofilaremic with pathology. Pathology is primarily dermal with inflammatory manifestations such as Calabar swellings. These swellings appear and disappear and seem to be brought on by exertion. Complications involving kidney, heart and lung disease occur less frequently. Other manifestations include elevated white cell counts, an increase in total immunoglobulin levels, and an increase in antifilarial IgG and IgE.

Diagnostic techniques include Giemsa stained thick and thin blood films, membrane filtration and Knott's concentration. In the latter, one milliliter of blood can be collected in formalin and stored for later examination. These parasites are diurnally periodic and blood must be collected between 1200 and 1600 hours. Microfilaria counts are very low or negative at night. DEC is a macrofilaricidal agent for Loa and can be used in diagnosis. Following DEC treatment, subcutaneous nodules develop involving dying adult worms. DEC is an effective chemotherapeutic agent as demonstrated by a drop in antifilarial IgG and clinical improvement. DEC also works as an effective prophylactic agent. In a double-blind study in West Africa, 10% of Peace Corps volunteers who received a placebo became infected with Loa, while none of the volunteers treated prophylactically with DEC became infected.

2.1.2. Onchocerciasis in Guatemala--Dr. J. O. Ochoa

More than 50,000 individuals in Guatemala are infected with Onchocerca volvulus. Transmission occurs in 4% of the geographical area of the country. These areas lie in the foothills of the Sierra Madre near the Pacific Ocean at an elevation between 600 and 1500 meters. January to April (the rainy season) is the critical period of transmission. An ivermectin trial was initiated in Guatemala in 1988 and is ongoing. In order to evaluate the effect of repeated doses of ivermectin on transmission, data were collected over an 18 month period (1988-1989) in four villages. One purpose of the study was to develop recommendations for optimal use of ivermectin in Guatemala. During the time of this study, microfilaria levels in these villagers were seen to drop when compared to pre-treatment levels. A significant decrease was also observed in the number of L3 larvae in the vector population. Thus, ivermectin was shown to have a significant impact on the transmission of O. volvulus in Guatemala. Such a significant impact has not been seen in similar studies in West Africa. One reason for this may be that an infected fly in West Africa may carry 15 larvae, while in Guatemala it may carry only one or two.

2.1.3. Onchocerciasis in Africa--Dr. Stefanie Meredith

The vectors of Onchocerca volvulus have a broader distribution in Africa than the parasite itself. While little is known about the importance of zoophily in East Africa, its importance in West Africa is well-established. The form of O. volvulus that causes blindness is almost always associated with savannah areas, while the non-blinding form is usually found in forest areas. Exceptions are the severe blinding forms seen in forest areas of Zaire and Sierra Leone. In the Onchocerciasis Control Programme in West Africa, occasional outbreaks of disease are seen due to infected flies migrating into the O.C.P. from untreated areas.

All of the Simulium vectors that bite humans also bite animals, although some species may prefer to bite humans. Some of the vectors are unknown for a number of species of Onchocerca that infect animals. The problem is compounded by the fact that several other species of Onchocerca are easily confused with O. volvulus. Zoophily is not a problem in all areas of Africa, but it is a problem in many regions. Currently, species are identified by the dissection of flies, staining and microscopic examination. Species identification with certainty is difficult. In areas with high cattle populations relative to the human population, there is less transmission of Onchocerca to humans. There are proposals for mass ivermectin treatment programs in the O.C.P. area, and it will be important to monitor what happens to O. volvulus vector populations in these areas. Species-specific, sensitive DNA probes would be very useful to study the distribution of various Onchocerca species in the wide variety of potential Simulium vectors. Probes would also be useful in monitoring Onchocerca in vector populations in ivermectin treated areas and would help to address many of the problems of Onchocerca epidemiology discussed above.

2.1.4. Repeats of Onchocerca volvulus and Nodule Variation
Dr. Francine Perler

Three important factors to examine when discussing Onchocerca DNA probes are: 1) the need to differentiate Onchocerca volvulus from various animal species of Onchocerca in vectors; 2) the need to differentiate the blinding form of O. volvulus from the non-blinding form and 3) the need to identify as few as a single microfilaria in skin snips of humans. O. volvulus possesses several distinct repeat families with a copy number of hundreds to thousands. Contamination of Onchocerca nodule DNA with human DNA made precise copy number calculations difficult. Less than 5% of nodule DNA was Onchocerca DNA, greater than 95% was human DNA. Two different families of repeats were identified.

The clone pOV8 was found to contain a tandem repeat that cross-hybridized to all infectious parasitic nematodes tested. DNA from individual Onchocerca nodules was digested with various restriction enzymes, run on agarose gels and Southern transferred to nitrocellulose filters. When these blots were probed with pOV8, variation in the restriction pattern was seen between the samples from different nodules. The clone pOV26 was found to contain a member of an interspersed repeat family. This clone proved to be Onchocerca specific, but cross-hybridized to DNA from all of the various Onchocerca species tested. Both clones could be used to detect DNA from a single L3 larva, but neither clone was species-specific. The copy number for each of these repeats was found to be several hundred.

2.1.5. O. volvulus Specific DNA Probes--Dr. W. Piessens

The clone pOV3 was isolated from an O. volvulus genomic library and was found to be a member of a tandemly repeated DNA family. This clone showed no hybridization to Brugia, Dirofilaria, human or black fly DNA but did hybridize to O. volvulus DNA from both forest and savannah forms and to O. ochengi DNA. The hybridization to O. ochengi DNA is a problem since it can be transmitted by the same vectors as O. volvulus. This probe was sensitive enough to detect the DNA from a single L3 larva, but was not sufficiently species-specific. Sequencing the clone revealed two copies of a 149 base pair repeat element. Different members of this repeat have been cloned by various research groups. The different members of this family have demonstrated different degrees of specificity in DNA hybridization assays.

2.1.6. Oligonucleotide Probes for *O. volvulus*--Dr. William Harnett

An *O. volvulus* library was constructed in gt10 using DNA from a Liberian isolate. Differential screening with *O. volvulus* DNA, *O. gibsoni* DNA, *O. guttorosa* DNA and human liver DNA was used to select an *O. volvulus* specific probe from about 80,000 clones. This clone, C1A1, gave 50-fold better hybridization to *O. volvulus* genomic DNA than to *O. gibsoni* genomic DNA. It hybridized to DNA from both forest and savannah strains of *O. volvulus*. In order to improve the specificity, C1A1 was subcloned and sequenced. Based on these data, two oligonucleotide probes were synthesized, C1A1/1 and C1A1/2. The latter gave the best specificity, with only slight cross-hybridization to *O. gibsoni* (300-fold less than to *O. volvulus*). This oligonucleotide could detect 2 ng of *O. volvulus* DNA in dot blots with a 24 hour exposure. The DNA from about five L3 larvae could be detected with a 48 hour exposure. This probe belongs to the same family of *Onchocerca* repeats that has and will be described by other investigators.

2.1.7. Repeats in Different Species of *Onchocerca*--Dr. A. J. Trees

In designing DNA probes that are species-specific for *Onchocerca volvulus*, cross-hybridization is most often a problem with DNA from *O. gibsoni*. However, since these two species are not sympatric, this is only an academic problem. The fact that *O. volvulus* and *O. gibsoni* each have a haploid chromosome number of four, while other *Onchocerca* species have a chromosome number of five, is a further indication that these two species are very closely related.

pOA1 contains a 144 base pair tandemly repeated DNA sequence from *O. armillata*. This repeat is not related to the 149 base pair repeat seen in *O. volvulus*. Due to contaminating human DNA in *O. volvulus* DNA libraries, a high percentage of the clones hybridized to human DNA and were probably human DNA clones. The clone pOV2 was shown to be specific for *O. volvulus* under stringent conditions (except for some hybridization to *O. gibsoni*). With pOV2 as a probe, as little as 0.37 ng of parasite DNA could be detected in dot blots. Since one *Onchocerca* microfilaria has about 300 pg of DNA and one L3 has about 1.1 ng of DNA, this probe should be useful in detecting single L3 larvae. This difference in the amount of DNA between microfilariae and L3 larvae correlates well with the observed difference in cell number of these two stages (an L3 has about three times as many nuclei as a microfilaria). The clone pOV5 was found to hybridize only to a savannah isolate from Mali, and so may be a candidate for a savannah specific probe. This clone does not hybridize to any of the 149 base pair repeat clones being studied by various groups.

The use of DNA probes to detect L3 larvae in vectors depends on the ability to break open L3 larvae so their DNA can be bound to a filter for hybridization. A wide variety of methods have been tested for breaking open L3 larvae, but none are very satisfactory

other groups have also had difficulty releasing DNA from L3 larvae. Physical disruption of the worm may be the most efficient and consistent approach. Whatever method is used to break open the worms, it should be compatible with dissection of the vectors on microscope slides. A method that would enable a researcher to first dissect the fly and then hybridize the L3 larvae with a DNA probe would be ideal. Such a method would enable researchers to confirm or refute morphometric analysis. DNA probes could be used to validate new morphometric methods for distinguishing species and strains of Onchocerca.

2.1.8. Repeats from Savannah O. volvulus--Dr. Stefanie Meredith

An O. volvulus library was constructed using DNA from a savannah isolate from Mali. In isolating a species-specific clone, cross-hybridization to O. gibsoni DNA was the biggest problem. The species-specific clone isolated was found to hybridize to O. volvulus isolates from both Africa and America. The clone was found to contain 12 copies of the 149 base pair O. volvulus repeat. Breaking open L3 larvae so they can be detected with the DNA probe presents a continuing challenge. Perhaps if a little L3 DNA can be released from the vector, then the polymerase chain reaction could be used to amplify the repeat DNA for detection. Some success was obtained when L3 larvae were repeatedly frozen in liquid nitrogen, thawed at between 30 and 50 degrees centigrade, and then incubated for at least three hours in dithiothreitol and Triton X-100. It will be necessary to devise simpler and more efficient methods for lysing L3 larvae in the future.

2.1.9. Forest and Savannah Specific O. volvulus DNA Probes
Dr. Thomas Unnasch

Two possible uses for O. volvulus DNA probes are to identify species and to identify strains of the parasite. For example, in the Onchocerciasis Control Programme region in West Africa, it will be important to identify the source of outbreaks of Onchocerca. It may be important to determine whether black flies are carrying forest forms coming from outside the OCP boundary, or if they are carrying savannah forms that escaped elimination within the OCP region.

The clone pFS1 was isolated from a library made with DNA isolated from a Liberian forest O. volvulus isolate. Under stringent conditions, this clone was found to hybridize only to forest isolates and not savannah isolates. The single exception is that pFS1 hybridized to a forest/savannah transitional form found in a savannah region of Togo. pFS1 contains an O. volvulus repeat that belongs to the 149 base pair tandem repeat family mentioned by others. This family exists in about 4000 copies per haploid genome. When hybridized under stringent conditions, the pFS1 clone hybridizes to a subset of the repeats of this family (3%-5%). This hybridization to only a subset of the repeats is

what makes the clone forest specific, but it also reduces the sensitivity of the clone. The clone was found to be forest-specific when a hybridization temperature of T_h -10°C was used, but was not specific at T_h -13°C.

A savannah specific clone, pSS1, does show some weak cross-hybridization to Onchocerca forest DNA when large amounts are loaded on a Southern blot. This clone was sequenced and found to contain two copies of the 149 base pair repeat. The sequence was examined for differences with forest repeat DNA. A region of difference was identified and sub-cloned. This sub-clone did not hybridize to forest DNA when stringent conditions were used (T_h -9°C to T_h -15°C). As the temperature of hybridization was decreased over this range, the sensitivity improved from 100 ng to 3 ng of savannah O. volvulus DNA detected. At hybridization temperatures below T_h -15°C, cross-hybridization to forest O. volvulus was observed.

Future work with these DNA probes will include testing additional forest, savannah and transitional forms of O. volvulus. The DNA probes themselves must be improved so that sensitive detection can be achieved over a broader range of temperatures without sacrificing species-specificity. This will be necessary for the assay to prove useful in endemic countries.

2.1.10. DNA Probes for Loa loa--Dr. Thomas Nutman

A library was constructed from DNA isolated from Loa loa microfilariae. This Loa DNA was found to contain 5-8% human DNA contamination. Three clones (LL1, LL2 and LL3) were selected for further analysis. All three of these clones are related as shown by DNA hybridization. LL2 and LL3 were found to have identical restriction maps. LL3 was found to be very species-specific and did not cross-hybridize to DNA from any other species of filaria tested. Sensitivity using a non-radioactive chemiluminescent detection system was 40 to 80 pg, less than the amount of DNA in a single microfilaria. Individual microfilariae in blood samples were detected by chemiluminescence using the blood blot method of Poole and Williams (C.B. Poole and S.A. Williams, Mol. Biochem. Parasitol. 40, 129-136, 1990).

2.1.11. Repeated DNA Probes for Loa loa--Dr. Thomas Egwang

The clone pLL20 was selected from a genomic library of Loa DNA. The 3.9 kilobase insert was sub-cloned into the plasmid vector pBR322. The limit of sensitivity with this clone was 2.5 ng of Loa DNA. The species-specificity of the probe was acceptable, since cross-hybridization was seen only with Brugia malayi DNA (ten-fold less than to Loa DNA). No cross-hybridization was observed with O. volvulus DNA. The probe was successfully used to detect Loa DNA in the vector, although it is very difficult to lyse the L3 larvae. The probe was also used to detect

microfilariae in blood. For this to work, the blood first had to be treated with Proteinase K and Triton X-100 before it could be filtered through nitrocellulose. For squash blots of the Chrysops vector to be practical, major improvements will need to be made. The vector is very large, and it is not practical to simply squash it on to nitrocellulose (most of the material simply washes away). It is also not practical to isolate DNA from each individual fly. Simple and effective means for lysing L3 larvae must be derived. Finally, the assay must be perfected using non-radioactive probes.

2.2. Diagnosis of Lymphatic Filariasis

The second scientific session of the Workshop on DNA Diagnostics and Filariasis was chaired by Dr. C.P. Ramachandran.

2.2.1. Classical Methods for Identification of Lymphatic Filarial Parasites-- Dr Purnomo

The three species of filarial parasites that infect humans in Indonesia are Brugia malayi, Brugia timori and Wuchereria bancrofti. Using morphological measurements and anatomical markers, the microfilariae, larvae and adults of these species can be distinguished. The characteristics that can be used to differentiate the microfilariae include the mean length of the worm, the length versus width of the cephalic space, Giemsa staining of the sheath, number of terminal nuclei in the tail, arrangement of the body nuclei, and Innenkorper length. The best way to differentiate L1 larvae is to examine the shape of the tail and to look for the presence of tail nuclei. The best method for distinguishing the L2 larvae is to examine the shape of the tail. For L3 larvae, the most important distinguishing characteristics are the shape of the caudal papillae. The distance from the anal pore to the tip of the tail is also important. Female adult worms can be distinguished by the length of the body, the shape of the head, the length of the esophagus, and especially the position and measurement of the ovijector. The adult male worms can be differentiated based on the length of the worm, the number of pairs of anal papillae, the ratio of the length of the left spicule compared to the right spicule, and the dimensions of the Gubernaculum.

Brugia malayi and Brugia pahangi can be distinguished by examining the Innenkorper length of the microfilariae, the length of the left spicule in adult males, and the distance from the vulva to head and ovijector length in adult females. The new species of Wuchereria (kalimantani) can be easily distinguished from W. bancrofti. The left to right spicule ratio can be used to distinguish the adult males; while body length, Innenkorper length, and length to width ratio of the cephalic space can be used to distinguish the microfilariae. Thus, a trained microscopist can differentiate the species based on morphometric measurements. These methods are, however, difficult and time-consuming.

2.2.2. Wuchereria bancrofti in India--Dr. Pani

In India, 30% of the districts have never been surveyed for filariasis so the full extent of the disease is unknown. A nocturnally periodic form of B. malayi is found in three pockets in India, while W. bancrofti is found in a majority of the districts. Chemotherapy and integrated vector management programs have been used to contain the disease in many parts of the country. Because of the massive difficulties in carrying out thorough epidemiological surveys in a country as large as India, the impact of chemotherapy and integrated vector control management programs is difficult to assess. Computer simulation has predicted that it will take 50 years to eliminate transmission of W. bancrofti by vector control alone. Studies have shown that conventional methods of surveillance may underestimate the proportion of infected individuals by 30-50%. There is a great need to collect accurate epidemiological data all over the country and to assess the effectiveness of control programs in specific regions. DNA probes should provide a way to improve the current epidemiological methods.

2.2.3. Bancroftian Filariasis in China--Prof. Zheng Hui Jun and Dr. W. Piessens

China has a population of 1.1 billion people. 40% of these people live in areas that are currently or were once endemic for filariasis. There are currently 200-300 million individuals at risk and 20-30 million infected. The range of W. bancrofti extends from southern China north to Korea. The range of B. malayi extends from Vietnam in the south to the coast near Formosa farther north. A concerted effort to eliminate the disease involved vector source reduction and annual treatment with DEC. DEC was given in mass treatment in some areas and by distribution of medicated salt in others. A coverage of 85-90% was achieved in these mass treatments. In one province, the frequency of microfilaria positive individuals was reduced from 16% to less than 1%. Some individuals developed recurrent microfilaremia even after five to six courses of DEC treatment. Some of these individuals have been effectively treated with ivermectin.

There is a need for accurate and efficient means to monitor the success of parasite control programs. An informed decision needs to be made on when to stop the control measures. In order to do this, methods are needed that can be used to screen millions of samples. These methods must provide species-specific information, since workers dissecting mosquitoes in the field often confuse B. malayi, B. pahangi and W. bancrofti. It is hoped that DNA probes will be able to make a significant contribution in the future collection of accurate epidemiological data. In order to do this, DNA probes must be able to accurately monitor low-level transmission in control program areas.

2.3. DNA Probes for Wuchereria and Brugia

The third scientific session of the Workshop on DNA Diagnostics and Filariasis was chaired by Dr. C.P. Ramachandran.

2.3.1. B. malayi and B. pahangi Specific Oligonucleotide Probes

Dr. Steven A. Williams

The repeat DNA family with the highest copy number in the Brugia genome is the Hha I repeat. Initially, 15 Hha I repeats were cloned and sequenced from B. malayi and 15 from B. pahangi. In both species, the repeat is 322 base pairs in length and exists in 30,000 copies per haploid genome. When the sequences of two repeats from the same species are compared, they are found to differ in about 3% of the nucleotide positions. When repeats are compared between the two species, 11% of the nucleotide positions are different. When cloned copies of this repeat family are radioactively labeled and used as hybridization probes, 100 pg of Brugia DNA can easily be detected (this is less than the amount of DNA found in a single microfilaria or L3). Because of the 89% sequence homology between the two species, a B. malayi repeat will cross-hybridize to B. pahangi DNA and vice-versa. These labeled repeats can be species-specific if hybridization is done using very stringent conditions. However, under these conditions of extreme stringency, sensitivity is decreased.

In order to improve the specificity of these probes without sacrificing sensitivity, oligonucleotides were synthesized based on the region of the Hha I repeat that is most different between the two species. In this 50 nucleotide long region, the repeats are only 70% homologous, while the rest of the repeat is 93% homologous. The short oligonucleotides synthesized were a 29-mer specific for B. malayi and a 21-mer specific for B. pahangi. These oligonucleotides can detect 100 pg of DNA in a species-specific manner under conditions of moderate stringency.

Detection of Brugia L3 larvae in mosquitoes has been accomplished using three different methods. First, L3 larvae were detected using the Brugia-specific probe following incubation at 95°C for 30 minutes in 0.3N NaOH and 6M urea. Second, DNA from L3 larvae were also detected following mechanical homogenization of the worms and digestion with proteinase K, beta-mercaptoethanol and Sarkosyl for three to four hours at 60°C. Finally, PCR has been used to successfully amplify repeats from infected mosquitoes following mechanical homogenization and boiling for one hour.

We have developed an assay that can be used to detect individual microfilariae in blood using the oligonucleotide probes. The blood sample is filtered through nitrocellulose with a pore size of 5 μ m. The filter is then rinsed with NaOH and the microfilariae are digested by soaking the filters on stacks of filter paper saturated with proteinase K, beta-mercaptoethanol and

Sarkosyl. This method lyses both *B. malayi* and *B. pahangi*. A simpler method that lyses only *B. malayi* microfilariae is to replace the proteinase K step with NaOH. It is not known why this does not work for *B. pahangi* microfilariae. Using this "blood blot" method, individual worms have been detected using radioactively labeled oligonucleotide probes and using non-radioactive chemiluminescent detection. This "blood blot" method has been field tested and will be discussed in a talk by Dr. Felix Partono. The oligonucleotide probes have also been labeled with biotin and tested extensively. These results will be discussed by T. Supali. Finally, these probes have been used in conjunction with PCR to detect very low-level infections in Indonesia.

Future work with these probes will focus on improving their sensitivity using non-radioactive methods. Field studies in a variety of locations will be needed to validate the use of these non-radioactive probes. The use of PCR with these probes also deserves further investigation. Finally, it would be useful to develop an L3 stage-specific DNA probe designed to detect an L3 specific RNA.

2.3.2. Non-Radioactive DNA Probes for Diagnosis of Brugian Parasites---Dra. Taniawati Supali

Different species and strains of filarial parasites are often difficult to differentiate by morphology. DNA probes could prove useful to supplement or replace morphological examination of blood smears in the field if a practical assay can be developed. An important element in the development of a practical assay is the replacement of radioactive probes with non-radioactive probes. In this study, the species-specific oligonucleotide probes for *B. malayi* and *B. pahangi* already discussed by Dr. S.A. Williams were used. These oligonucleotide probes were modified by the addition of biotinylated uridine residues to either the 5' end or to both the 5' and 3' ends. Three types of biotinylated probes were evaluated. The first had one chain of 30 biotinylated uridines on the 5' end. The second had one chain of 30 biotinylated uridines on the 5' end and another chain of 30 on the 3' end. The third had one chain of 90 nucleotides added to the 5' end. This chain had 45 biotinylated uridine residues alternated with 45 non-biotinylated thymidine residues. The last probe proved to give the best sensitivity in dot blot hybridization assays.

The probes were hybridized to dot blots containing doubling dilutions of phage lambda DNA (negative control), *Hha* I repeat DNA (positive control), *B. malayi* DNA, and *B. pahangi* DNA. The spots had from 12.5 ng of DNA down to 100 pg. Hybridizations in solution containing no formamide were done in separate experiments at temperatures ranging from 30°C to 80°C in 5°C increments. The probes were also tested in hybridizations containing 50% formamide at 25, 30, 35, 37, 42, and 47°C. Probe DNA was detected in these experiments using a streptavidin-alkaline phosphatase detection system. In experiments with and without formamide, the best

sensitivity was seen at 30°C (the 100 pg spots were easily detected). At this reduced stringency, however, cross-hybridization was evident. To completely eliminate cross-hybridization, 75°C was necessary without formamide. This temperature is not convenient for doing hybridizations. Species-specificity using 50% formamide was obtained at 35°C with the B. malayi probe and at 37°C with the B. pahangi probe. At these temperatures, the limit of sensitivity of the biotinylated oligonucleotides was about 400 pg.

The biotinylated oligonucleotide probes show promise for use in endemic nations. These probes are species-specific, reasonably sensitive, extremely stable, convenient to use, and relatively inexpensive.

2.3.3. Repeat DNA Probe for B. malayi--Dr. W. Piessens

The first Brugia probe cloned was a ribosomal DNA clone that was not species-specific. A second clone developed was pBm15 which was found to contain copies of the Hha I repeat already discussed in the previous talk. This clone was used to develop an assay for detecting L3 larvae in mosquitoes. This method lyses the L3 larvae using only proteinase K and SDS. In one study, mosquitoes were fed on a B. malayi infected cat and two weeks later were dissected and examined for L3 larvae. Next, the dissection fluid from each mosquito was spotted directly onto nitrocellulose filters. Approximately 150 mosquitoes were tested and 98% of infected mosquitoes were properly identified. The number of larvae in a mosquito as seen by microscopy was proportional in most cases to the radioactive signal obtained with the DNA probe. To process hundreds of thousands of mosquitoes in large epidemiological studies, a faster and simpler assay is necessary. "Squash blots" were developed in which the mosquito is squashed onto nitrocellulose and then treated with proteinase K, chitinase and detergent. In one study, the proportion of infected mosquitoes in a population as measured by dissection was the same as that measured using the "squash blot" method.

2.3.4. W. bancrofti DNA Probes--Dr. Nithya Raghavan

A genomic W. bancrofti DNA library was constructed in gt11 using DNA isolated from Indian microfilariae. 50,000 recombinants were screened for repeated DNA sequences using total genomic W. bancrofti DNA. The inserts from the four clones giving the strongest hybridization signal were subcloned into the plasmid vector pGEM-Z for further analysis. The four inserts were found to have the following insert sizes: clone 1, 6.6 kb; clone 2, 5.2 kb; clone 3, 3.2 kb; and clone 6, 5.0 kb. At moderate stringency none of the cloned inserts hybridized to one another. The restriction maps of the four clones also showed no similarity. Therefore, these clones belong to different families or sub-families of W. bancrofti repeats.

The species-specificity of these clones was examined in duplicate dot blots which had 20 ng of B. malayi, B. pahangi, W. bancrofti and human DNA applied to nitrocellulose filters. Clones 1, 2, and 6 hybridized only to W. bancrofti DNA. Clone 3 was not tested. Hybridizations were done to check the sensitivity of each clone and to estimate the copy number. The most sensitive clone could detect 10 pg of W. bancrofti DNA and had an estimated copy number of about 8,000. Clones 1, 2 and 6 were hybridized in Southern blots with W. bancrofti clones Steve Williams has isolated from India and Indonesia. Both of Steve Williams' clones hybridized to clones 1, 2, and 6 but not clone 3. Clone 1 hybridized significantly better than clones 2 and 6. These results indicate that all of these cross-hybridizing repeats may belong to the same repeat DNA family.

Repeat DNA sequences may not be the only sequences useful as DNA probes. A single copy gene might also prove useful if the target DNA can first be amplified by PCR. To demonstrate this, a single or low-copy number gene coding for a W. bancrofti antigen was used. This clone hybridizes to W. bancrofti and B. malayi DNA, but not to the DNA of other filarial parasites or human DNA. Oligonucleotide primers that hybridize to this gene were used to successfully amplify DNA from 1, 10, and 100 W. bancrofti microfilariae. It was demonstrated that these primers could successfully amplify as little as 10 pg of W. bancrofti genomic DNA. DNA from these amplified samples could then be easily detected with the antigen gene clone. Thus, single copy genes may also prove useful as DNA probes when coupled with the PCR technique.

2.3.5. Repeat DNA Families in W. bancrofti--Dr. Steven A. Williams

We have constructed three DNA libraries for W. bancrofti using microfilariae from Indonesia, Egypt and India. Additionally, we have made a W. kalimantani DNA library from microfilariae from Kalimantan, Indonesia. These libraries have been constructed in the plasmid vector pBluescript and in the bacteriophage vector M13. Since all of the libraries were made with microfilariae samples contaminated with some human cells, differential screening was used to select W. bancrofti repeat DNA clones. The libraries were first screened with the W. bancrofti total genomic DNA (contaminated with human DNA), and then counterscreened with total genomic human DNA. Only clones that hybridized strongly to the W. bancrofti DNA and not at all to the human DNA were selected for further analysis. These repeats were shown to have a copy number on the order of a few thousand, and could detect 100 pg of W. bancrofti DNA in dot blots.

All of the repeats selected from the three isolates of W. bancrofti were shown to hybridize to one another at moderate stringency. They also hybridized to three out of four Indian W. bancrofti clones obtained from Dr. Nithya Raghavan. At high

stringency, however, any given repeat clone hybridized to only a subset of the repeat clones. Thus, at moderate stringency, all of the repeats cross-hybridize and clearly are related (i.e. belong to the same repeat family). High stringency hybridizations show that individual repeats belong to different sub-families. Within a sub-family (those repeats that hybridize at high stringency) the repeats may show greater than 95% homology. Repeats from different sub-families (those repeats that hybridize only at moderate stringency) may show only 80% homology. Thus, these repeats probably evolved from a single ancestral repeat family, that later diverged into many different sub-families. Because of this, it is extremely difficult to obtain an accurate estimate of the copy number for these repeats. The number obtained will depend on the stringency used in the hybridizations.

The members of the repeat family described here are dispersed and not organized in tandem. There are two lines of evidence to support this assertion. First, Dr. Nithya Raghavan has shown that when W. bancrofti genomic DNA is digested with various restriction enzymes, run on a gel, Southern transferred to nitrocellulose, and hybridized with cloned repeat DNA; that the entire smear of genomic DNA hybridizes. This suggests that the repeat lies on a random selection of the genomic DNA fragments found in these digests. Second, experiments done in my laboratory have shown that when repeat inserts are purified from the vector and hybridized to one another, that only a central region of the fragment hybridizes and that the flanking regions are unrelated. It is highly probable, therefore, that the members of this repeat family in W. bancrofti are arranged as a dispersed type of repeat. The arrangement and position of these repeats will be compared between the three different geographic isolates of W. bancrofti. The study of the genetic organization of these repeats in W. bancrofti should prove to be a fascinating problem and may provide clues as to how these sequences can be best exploited for use as DNA probes.

2.3.6. W. bancrofti Probes from Sri Lanka--Dr. Eric Karunanayake

A genomic DNA library of Sau 3A digested Sri Lankan W. bancrofti DNA was constructed in the bacteriophage lambda vector EMBL 3. The genomic DNA was prepared from microfilariae that were contaminated with some human cells. Density gradient ultracentrifugation was used to separate the W. bancrofti DNA from the human DNA. Because filarial parasite DNA has a lower fraction of GC base pairs than does human DNA, the parasite DNA has a lower density than the human DNA and will band higher in the gradient. Despite these precautions, the EMBL 3 library was screened with both W. bancrofti total genomic DNA and human total genomic DNA. Of 15,000 recombinant clones screened, 8 showed strong hybridization with the parasite DNA and no hybridization with the human DNA. One clone, EMBL 3/Wb 34 with an insert of 16.1 kilobases, was selected for further characterization. When this clone was radioactively labeled and used as a probe in dot blot

hybridization assays, no cross-hybridization was seen to human DNA, mosquito vector DNA, or to DNA from a cattle filarial parasite found in Sri Lanka. The limit of detection of W. bancrofti genomic DNA with this probe was shown to be 25 pg. This probe needs to be tested against other filarial parasite DNA, but it appears to be species-specific and quite sensitive.

2.3.7. DNA Probes for W. bancrofti-- Dr W. Piessens

A W. bancrofti genomic library was differentially screened with W. bancrofti genomic DNA and a mixture of Brugia, Setaria, human and mosquito DNA. One clone that hybridized strongly to W. bancrofti DNA but not to the mixture of DNA from different species, was selected for further analysis. When this clone (clone 35) was used to hybridize to genomic digests on Southern blots, a pattern was seen that suggested an interspersed repeat rather than a tandem repeat. The specificity and sensitivity of this clone was tested in dot blot hybridization assays. Hybridization to two W. bancrofti isolates from China and one from Sri Lanka was observed. No hybridization was seen to Brugia DNA or to the DNA of other filarial parasites. When radioactively labeled, the clone was sensitive enough to detect the DNA from a single L3 or about five microfilariae. When various non-radioactive methods of detection were tried, the sensitivity of the clone dropped 10 to 1000-fold.

To improve the sensitivity of the assay, PCR primers were synthesized that hybridized to different regions of the repeat. One pair of primers amplified a region of the repeat that was specific for W. bancrofti. B. malayi DNA was not amplified with these primers. A second pair of primers amplified a region that was partially specific (only a little amplification of B. malayi DNA), while a third pair of primers gave no specificity (amplified both W. bancrofti DNA and B. malayi DNA). The most specific primers could amplify as little as 10 pg of W. bancrofti DNA, while not amplifying as much as 200 ng of B. malayi DNA. Recent work has demonstrated that L3 larvae digested with proteinase K can be put into the PCR reaction and their repeats can be amplified. This PCR amplified material can then be detected with non-radioactive DNA probes. Mosquito material that has been crushed, digested with proteinase K and extracted can be put into PCR reactions as well. When this is done with infected mosquitoes, parasite repeat DNA is sometimes amplified. Research is proceeding to make this assay as practical as possible for use on large numbers of mosquitoes.

2.4. Field Application of DNA Probes

The fourth scientific session of the Workshop on DNA Diagnostics and Filariasis was chaired by Dr. Thomas Egwang.

2.4.1. The Use of PCR and Chemiluminescence for Parasite DNA Detection--Dr. Steven A. Williams

The polymerase chain reaction (PCR) can be used to specifically amplify target DNA sequences recognized by a DNA probe. Such amplification can increase the sensitivity of a DNA detection assay by at least several hundred thousand-fold. The main difficulty in implementing non-radioactive labeling and detection of DNA probes is that these methods are generally less sensitive than radioactive methods. PCR provides the means to amplify the target parasite DNA, so that less sensitive non-radioactive probes can detect individual parasites. We have devised a method that combines PCR amplification of the Brugia HhaI repeats with detection of peroxidase labeled DNA probes by chemiluminescence.

This method was tested on 50 blood samples collected from humans and cats in Tanjung Pinang, Indonesia. Many of these blood samples contained very low numbers of microfilariae, two had only two microfilariae per 60 ul of blood. The blood samples were digested with proteinase K and then extracted with phenol and chloroform. From each 100 ul blood sample, 2 ul was removed for PCR amplification. Two primers that hybridize on either side of the divergent region of the Hha I repeat were used for amplification. The primers were annealed at 50°C, polymerization was carried out with Tag polymerase at 72°C, and DNA strands were denatured at 95°C. The 50°C annealing temperature gives the reaction greater specificity than the typical 37°C annealing. After 25 cycles, 5 ul samples were removed from each reaction and run on agarose gels to check for amplification of the Hha I repeats. When amplification was successful, bands representing the amplified repeat DNA were easily visualized following staining of the gel with ethidium bromide. All of the samples that contained Brugia microfilariae (as shown by morphological examination) were successfully amplified by the PCR.

Two microliters of each PCR reaction were then spotted on a dot blot. The amount of PCR product loaded on the blot represented 0.04% of the original blood sample (2 ul of the 100 ul blood sample was put into the PCR reaction, and then 2 ul of the 100 ul PCR reaction was loaded on the dot blot). The rest of each digested blood sample (98 ul) was spotted onto another dot blot labeled "non-PCR". When this non-PCR blot was hybridized with the radioactive B. malayi and B. pahangi probes, faint spots were observed on the blot where the samples with the highest microfilaria levels were spotted. These results were obtained after the blot had been exposed to X-ray film for 3 hours. After two days, spots could be seen on all of the positive blood samples

(except the samples with only two microfilariae). The blots with the 2 ul of PCR amplified material were hybridized with three different probes. First, with radioactive probes, all of the positive samples were detected after only a three hour exposure. Second, with the biotinylated oligonucleotide probes discussed earlier by T. Supali, all of the positive samples were detected after about a four hour detection procedure. Third, with the peroxidase labeled oligonucleotide probes, all of the positive samples were detected with chemiluminescence after only a 15 second exposure! Thus, with all three different probes, all of the positive samples were correctly identified and there were no false positives.

Of the three detection systems used above, the chemiluminescent system is the simplest and fastest. The DNA probes are labeled in a simple one-step 15 minute labeling reaction. In the presence of glutaraldehyde, the horseradish peroxidase enzyme is coupled directly to the DNA probe. Following hybridization and washes, the probe is detected using luminol in the presence of hydrogen peroxide. The luminol is cleaved by the peroxidase to form 3-aminophthalate which is unstable and emits light upon decay. This light is detected by very brief exposures of the filters to X-ray film.

PCR coupled with non-radioactive detection may not be suitable for mass screening programs, but there are several possible uses for the technology. First, the method may be useful in mass screening of vector populations. Large numbers of mosquitoes could be collected from different sectors of a region being surveyed. Mosquitoes from each sector could be mashed and digested to release DNA from any filarial parasites that may be present. A small sample of the resulting supernatant could then be PCR amplified and screened with the non-radioactive DNA probe. Any sectors that were shown to have parasites could then be examined with a more detailed study. Second, the method could be used to screen for individuals with very low numbers of microfilariae in their blood. Since each microfilaria contains between 50 and 100 million *Hha* I repeats, just one microfilaria in one milliliter of blood would have 50,000 to 100,000 repeats per ul. It should be no problem to amplify this number of repeats with PCR. Third, the method could be used to screen body fluids or tissues for free DNA released by killed parasites. Four, the method could be used to enhance the sensitivity of probes which may be very species-specific but not very sensitive.

2.4.2. Species Diagnosis of Microfilariae in Blood Samples Using DNA Probe and Conventional Methods of Detection
Dr. Felix Partono

This study was undertaken to confirm or refute the report of Palmieri, et al., that B. pahangi was found to infect humans in South Kalimantan, Indonesia. The study was also used to field test the B. malayi and B. pahangi specific oligonucleotide probes in the blood assay described by Dr. Steven A. Williams in an earlier presentation. In this assay, individual microfilariae are detected as distinct spots on autoradiographic film. The DNA probes were compared in this study to the conventional microscopic method of morphological analysis. 200 human blood samples and 64 cat blood samples were collected. Blood samples from laboratory cats infected with B. malayi or B. pahangi were used as controls. From each blood sample, 270 ul were used for this study. 30 ul were used for blood smears, while the remaining 240 ul were divided into four 60 ul aliquots. Two of these were tested with the B. malayi and B. pahangi probes at the University of Indonesia, while the other two 60 ul samples were tested with the two probes at Steven Williams' laboratory in the U.S.A.

The 37 control cat blood samples containing only B. malayi microfilariae were all correctly identified by both the DNA probe and the morphological analysis. The 20 control cat blood samples containing only B. pahangi microfilariae were also correctly identified by both methods. The 200 microfilaria-positive human samples were all identified as B. malayi by morphological criteria. 193 of these were identified as B. malayi samples by the B. malayi probe. The seven samples judged to have B. malayi microfilariae by morphological analysis but judged negative by the DNA analysis, all had very low numbers of microfilariae. None of the human samples were identified as having B. pahangi by the morphological analysis. One of the human samples gave a faint spot and was scored as having one B. pahangi microfilaria by the B. pahangi specific probe. These data do not lend support to the idea that B. pahangi infects humans in South Kalimantan. Furthermore, the two methods showed 97% agreement, with the DNA probe apparently giving about 3% false negative results and 0.5% false positive results.

The 64 cat blood samples gave the following results by morphological criteria: 3 B. malayi, 37 B. pahangi, 22 D. repens and 2 no filariae. The DNA probes gave the following results: 5 B. malayi, 36 B. pahangi, and 23 no filariae. The DNA probes did not detect any signal in the D. repens samples. One of the samples judged as having a very low number of B. pahangi microfilariae by morphological criteria was identified as having one B. malayi microfilaria by the DNA probes. One of the samples identified as having no filaria by morphological study was identified as having one B. malayi microfilaria. The two methods showed 95% agreement. Thus, for both the human and cat blood samples, the only disagreements between the two methods were in samples that had very low numbers of microfilariae.

The number of B. malayi or B. pahangi microfilariae determined by the morphological analysis was correlated to the number of microfilariae determined by the DNA probes. The correlation was good, although more statistical analysis needs to be done on these data. The use of the B. malayi and B. pahangi oligonucleotide probes in an assay that detects individual microfilariae in blood was shown to be applicable for use on field collected samples. This assay is being refined and modified for use with non-radioactive DNA probes. Improvements need to be made to simplify the assay and to make it simpler before it can replace the standard morphological methods. We were not able to verify that B. pahangi infects humans in South Kalimantan, Indonesia.

2.5. Parasite and DNA Evolution

The fifth scientific session of the Workshop on DNA Diagnostics and Filariasis was chaired by Dr. Francine Perler.

2.5.1. Forest Versus Savannah O. volvulus--Dr. Thomas Unnasch

Onchocerca volvulus is believed to be a recent parasite of humans and therefore causes a lot of pathology. Most Onchocerca species are parasites of ungulates and cause very little pathology. Thus, O. volvulus has probably evolved relatively recently from an Onchocerca species that infected animals. Dr. Odile Bain has drawn a phylogenetic tree based on morphological data. Her tree shows that O. volvulus is closely related to O. ochengi, O. dukei and O. gibsoni. Techniques using primers to conserved regions of ribosomal RNA have been used by other groups to obtain direct sequence information from a variety of Onchocerca species. With data obtained from sequencing divergent regions of the rRNA, a phylogenetic tree can be drawn that looks similar to the tree established using morphological data. Here O. volvulus, O. guttorosa, and O. gibsoni are shown to be very closely related.

Differences in the forest and savannah strains of O. volvulus have been described since 1919. These differences include clinical differences, differences in the rate of blindness, differences in the transmission and development of the parasite in forest and savannah vectors, and allele frequency differences in isozyme analysis. We have used the 150 base pair tandem repeat family of Onchocerca to obtain sequence data from three isolates of O. volvulus. Well over 100 repeats have been cloned and sequenced from a forest isolate from Liberia, a savannah isolate from Mali, and an isolate from Zaire that shows characteristics of both types. A distance matrix analysis method was used to compare sequences from the three isolates. These data show a nucleotide substitution frequency within the isolates of about 10%. Between the isolates, the net substitution frequency is 26.5% Mali versus Liberia, 32.3% Liberia versus Zaire, and 17.2% Zaire versus Mali. The phylogenetic tree drawn from these data show that the Mali savannah

strain is more closely related to the Zaire strain than either are to the Liberian forest strain. The Mali savannah strain is a little more closely related to the Liberian forest strain than the Zaire strain is to the Liberian forest strain. Many different isolates of the strains need to be examined before firm conclusions can be drawn about the genetic relatedness of these types.

2.5.2. Evolution of Brugia in Indonesia--Dr. Alexandra S. Suwita

In collaboration with Dr. Steven A. Williams, Dr. Larry A. McReynolds, and Dr. Felix Partono data from 67 Hha I repeats cloned from various Indonesian isolates were examined. The data set consisted of only 322 base pair Hha I repeats sequenced in their entirety. The isolates compared were as follows: B. malayi zoophilic, B. malayi anthropophilic, B. timori, B. malayi from Tanjung Pinang, and B. pahangi. These repeats were all cloned in M13mp18 and sequenced by the Sanger dideoxy chain termination method.

The Hha I repeat consensus sequence was determined for each of the five isolates. The nucleotide differences between the consensus sequences were as follows: B. malayi zoophilic vs. B. pahangi 11.8%, B. malayi anthropophilic vs. B. pahangi 12.1%, B. timori vs. B. pahangi 11.5%, B. malayi zoophilic vs. B. malayi anthropophilic 2.8%, B. malayi zoophilic vs. B. timori 1.2%, and B. malayi anthropophilic vs. B. timori 2.8%. These data imply that the zoophilic strain of B. malayi is more closely related to B. timori than is the B. malayi anthropophilic strain. This is the exact opposite of what is predicted by morphological analysis done by Purnomo. His data shows that the anthropophilic strain of B. malayi is more closely related to B. timori than is the zoophilic strain. This apparent disagreement is based on consensus sequence differences that are much too small to be meaningful. In order to improve the analysis of the DNA sequence data, each repeat must be examined, not just the consensus sequences. In Dr. Steven A. Williams' talk on evolution, he will discuss such analyses.

The B. malayi Tanjung Pinang isolate is a very strange case. These parasites are from a cat collected in Tanjung Pinang and have been examined repeatedly by morphological analysis and shown to be B. malayi. However, in two separate cloning experiments, Hha I repeats from this strain have been shown to be much more closely related to B. pahangi than to any of the B. malayi isolates. It is possible that this isolate may represent a rare hybrid of the two species. Further samples will be collected from Tanjung Pinang and analyzed by morphology and DNA sequence in an attempt to answer this question.

2.5.3. Use of Repeat DNA to Examine the Evolution of Filarial Parasites--Dr. Steven A. Williams

Sophisticated computer methods are now available for the analysis of DNA sequence data for the purpose of drawing phylogenetic trees. These methods are more robust than methods that simply calculate the per cent difference between consensus sequences. In the talk just given by Dr. Suwita, she has shown that when our sequence data are analyzed by the simple consensus sequence method, the resulting tree disagrees with what is known about the biology of the parasites. Purnomo and Dr. Partono agree that based on various biological criteria, the anthropophilic strain of B. malayi should be more closely related to B. timori, while the zoophilic strain should not be as close to B. timori. Recall that the comparison of consensus sequences put the zoophilic strain closer to B. timori. These data have been reexamined using one of the most robust of the computer phylogenetic analysis programs. This method, known as P.A.U.P.---Phylogenetic Analysis Using Parsimony, can examine the relationships of all of the individual repeat sequences and enables the use of all of our DNA sequence data.

The phylogenetic trees drawn by this program give the following results. First, the B. timori repeats cluster together and cluster closer to the B. malayi anthropophilic cluster than to the zoophilic cluster. These three clusters are much closer to one another than they are to the B. pahangi cluster of repeat sequences. Thus, the results from this more robust analysis give a phylogenetic tree that is in agreement with the tree based on biology. The conclusions from these analyses are as follows: B. malayi and B. timori are very closely related and both are equally distant from B. pahangi. The zoophilic and anthropophilic isolates of B. malayi are very closely related, but their Hha I repeats do cluster separately from one another. Finally, repeats examined from periodic versus sub-periodic isolates of B. malayi do not cluster separately, but appear to belong to the same group. Thus, the sequence data supports Dr. Felix Partono's concept of anthropophilic versus zoophilic strains as a better way to differentiate B. malayi than periodic versus sub-periodic strains.

Highly repeated sequences are not the only useful sequences to examine for evolutionary analysis. Intervening sequences in genes or upstream and downstream non-coding sequences may prove to be as or more useful than repeats. We are currently sequencing the genomic copies of a 22 kD antigen gene (cloned in collaboration with Dr. Murray Selkirk). We are looking at intervening sequences between closely related strains while looking at coding sequences between distantly related species. Such analysis may provide a complete view of the evolutionary history of the filariae. This type of analysis is not simply of academic interest. The more detailed knowledge we obtain about the sequence and structure of highly repeated elements and other potentially useful clones, the more data we will have upon which to base our design of new, improved DNA probes.

3. RESULTS OF CODED DNA FILTERS

A variety of filarial-specific DNA probes have been developed. In order to expand our knowledge on the specificity and sensitivity of the existing probes coded DNA filters were sent to 11 different investigators. The filters, which were prepared at New England Biolabs by Catherine Poole, contained 67 different isolates representing about 30 different species or strains of filarial parasite. A list of the sources of the DNA is given in Table 3.1.

Since the DNA is very stable once it has been isolated and bound to nitrocellulose, it was possible to prepare the filters in one location and send them to different investigators in other parts of the world. Probes that used radioactivity were exposed to X-ray film and the intensity of the hybridization was subjectively rated on a scale of 1-4, with 0 being no hybridization, by the investigator. Four being the most intense hybridization. The results of the study were presented at the meeting in Jakarta, Indonesia.

3.1 Isolation of DNA and Hybridization of Filters

The DNAs were extracted from the parasites by digestion with proteinase K, SDS and EDTA [Emmons, S. W., Klass, M. R. and Hirsh, D. (1979) Proc. Natl. Acad. Sci. USA 76, 1333-1337]. The amount of DNA was quantified by agarose gel electrophoresis. The parasite DNA was stained with ethidium bromide and the intensity of fluorescence was compared to that of known DNA standards.

Each probe was tested at least twice, once under standard hybridization conditions, and once under specific hybridization conditions. The specificity and sensitivity of the probe varied depending upon the hybridization conditions. In general the specificity increased and the sensitivity decreased at higher stringencies of hybridization. By choosing standard conditions it was possible to group DNA probes that have related specificities. This information, when combined with the sequence data, allows identification of cloned sequences that are members of the same family of repeated DNAs.

The two conditions used for hybridization were: a) Standard Conditions; 5X SSC, 5X Denhardt's, 10 mM EDTA pH 8, 0.1% SDS and 100 µg/ml of sonicated and denatured DNA or b) Specific Conditions that were determined by each investigator. Some investigators used non-radioactive DNA probes that linked biotinylated DNA probe to avidin conjugated to

alkaline phosphatase. The degree of hybridization was determined by the color intensity of the chromogenic substrates. The probes used in the study are listed in Table 3.2

3.2 Results of the Coded Filters

Two different types of DNA filters were used; one with predominately O. volvulus DNA and the other with mostly DNA from lymphatic parasites. All major filarial parasites and control DNAs were included on both filters. The filter for the Onchocerca probes had a variety of different forest and savannah isolates of O. volvulus. The filter for the lymphatic probes had many different isolates of Brugia and W. bancrofti. Samples of the arrangements of the filters are given in 3.3. Abbreviations used in the results table are given in the list for the source of DNAs.

3.2.1 Brugia DNA probes

All of the Brugia probes used are members of the HhaI repeated DNA family. The oligonucleotide probes; BM 45, BP 4, BM 45B, BP 45B, BM L and BP L are synthesized probes that contain biotin. These oligonucleotides are synthesized to the species-specific region in the HhaI repeat [Williams, S., DeSimone, S., and McReynolds, L., (1988) Mol. Biochem. Parasitol. 28, 163-170]. The bound probes were detected by a chromogenic substrate which reacted with the alkaline phosphatase linked to the DNA probe by an avidin biotin bridge. Other probes used linkage of the DNA to peroxidase. These were detected by the photon release in the presence of luminol, substrate and enhancer which was detected on X-ray film.

All of the Brugia probes tested were very specific. No hybridization was observed to species other than Brugia. The B. pahangi probes hybridize strongly to B. pahangi with some slight hybridization to B. malayi on a few filters. The B. malayi probes hybridize to all B. malayi samples, those obtained from laboratory infections, and parasites obtained from Buton and Benkulu in Indonesia. The B. malayi probes also hybridize to B. patei and to B. timori. This suggests that these three species are more closely related to each other than they are to B. pahangi. This similarity does not present a diagnostic problem, for none of the three species exist in overlapping areas. B. malayi and B. timori are in different parts of Indonesia, and B. patei is found in East Africa.

3.2.2 W. bancrofti and Loa loa DNA probes

In contrast to the specificity of the Brugia probes, all of the W. bancrofti probes had some problem with cross-reactivity with other DNAs. The probe with the best specificity, EMBL3Wb34, hybridized strongly with the W. bancrofti samples with some hybridization with the Brugia samples. The other probes hybridized to other species of DNA including human DNA. The low amount of W. bancrofti DNA used on the filter [3 ng] and the possibility of human contamination in the sample reduced the sensitivity of the probes. It is likely that more characterization will be necessary before these probes can be used in the field.

Only one Loa loa probe was used, LL3. It gave a strong signal with Loa loa DNA but reacted slightly with B. malayi, D. immitis and Ascaris. It did not cross-hybridize with any of the Onchocerca samples.

3.2.3 Onchocerca DNA probes

Thirteen different Onchocerca DNA probes were tested against the samples on the filters. The probes showed a spectrum of specificities. Some, in stringent conditions, could distinguish forest and savannah forms of O. volvulus, while others cross-hybridized with a variety of filarial parasites. The probe pOV8 hybridizes to a wide variety of filarial parasites including B. malayi, D. immitis and Setaria. Five DNA probes cloned in the plasmids; pOV2, pOVI, pOV6, pOV3 and pOV8 are specific for the genus Onchocerca but not specific for O. volvulus. These probes also hybridize to O. qutturosa, O. ochengi, O. cervicalis as well as O. volvulus. None of these probes, however hybridized to non-Onchocerca species or to O. armillata.

pOA1, which was characterized as O. armillata specific, however, on the coded filter it identified only one of the three O. armillata samples and 1 of 33 O. volvulus samples. These unexpected results need to be further studied.

Three probes, pFS-1, pSS-1BT, pOVS134 and C1A1-2 have the greatest specificity of the probes tested. At stringent hybridization conditions they are all specific for O. volvulus or a subset of the O. volvulus samples. All four of the probes hybridize to some degree to the O. volvulus sample from Guatemala. However pOV3124 and pFSI-1 give a stronger signal. It will be interesting to have DNA sequence information to determine how the American parasite relates to its African ancestor. Three of the probes, C1A1-2, pOVS134 and pSS-1BT hybridize to three savannah type O. volvulus samples from Mali and Ghana. The probe pFS-1, the putative

forest specific probe, did not hybridize to any of these isolates.

DNA was isolated from 26 individual O. volvulus nodules in five different locations in Africa that contained different types of O. volvulus [Waninou- a forest and savannah area, and Koate, Abradinou, Nianda and Danane a forest type O. volvulus]. Of the 26 samples, pOVS134 recognized 20 of them, pFS-1 detect 10 and the other two probes C1A102 and pSS-1BT recognized 6 samples. Some samples were probably not detected because of low levels of DNA [i.e. Nianda group].

The probes pFS-1 and pSS-1BT only overlap on 2 samples from this group. There are 14 other samples that the probes recognize uniquely. The other probe, pOVS134 recognizes 20 samples in this group and has some overlap with both of the probes pFS-1 and pSS-1BT. Under the stringent conditions the probe C1A1-2 identifies only 6 samples in this group. Four of the six samples recognized by this probe are also recognized by pFS-1.

These results suggest that the O. volvulus probes can recognize different subsets of the O. volvulus samples. However, the increased sensitivity of the DNA probes has resulted in a decreased sensitivity. Under standard conditions pFS-1 recognized 24 samples from the villages which decreased to 10 under stringent conditions. The future challenge in developing this probes is to see if hybridization can correlate with blinding and non-blinding Onchocerciasis. The ability of the probes to distinguish O. volvulus from other species of Onchocerca has already been achieved.

The fact that forest and savannah "specific" probes hybridize to the same samples raises the possibility of mixed infections, or of interbreeding between different subspecies that results in a mixture of different sequences from this repeated DNA family. Most of the Onchocerca volvulus DNA probes are members of the same 149 bp repeated DNA family. This was determined prior to the meeting by comparison of the sequences of the cloned repeats. The probes pFS-1, pSS-1BT, pOVS134, pOV2, pOV5, pOV6 and C1A1-2 are related. All of these probes are specific for Onchocerca as a genus. Under the less stringent "standard" hybridization conditions, they all cross hybridize to a variety of Onchocerca [O. gibsoni, O. cervicalis and O. ochengi].

Table 3.1

<u>Filarial Parasites</u>	<u>Location</u>	<u>Source</u>
11 <u>O. volvulus</u>	Koate, Ivory coast of Africa (Forest)	M. Karam
22 "	"	"
64 "	"	"
115 "	"	"
220 "	"	"
44 "	Abradinou, Ivory coast of Africa (Forest)	"
92 "	"	"
161 "	"	"
170 "	"	"
173 "	"	"
400 "	Nianda, Ivory coast of Africa (Forest)	"
401 "	"	"
402 "	"	"
403 "	"	"
404 "	"	"
405 "	"	"
301 "	Danane, Ivory Coast of Africa (Forest)	"
304 "	"	"
305 "	"	"
306 "	"	"
307 "	"	"
<u>Control DNAs</u>		
Ascaris	USA	TRS Laboratories
C. elegans	"	L. McReynolds
<u>Non-filarial worms</u>		
pBR322	"	New England-Biolabs
M13	"	"
Blackfly (S. pictipes)	"	J. Lok
Mosquito (Ae. aegypti- Black-eye Liverpool strain	"	B. Christensen
Human (H)	"	L. McReynolds

The sources for the different parasitic samples are given in this table. The majority of the parasitic samples supplied from M. Karam represent only 1-3 adult O. volvulus parasites isolated from a nodule in one of five different West African villages. Some of the DNA samples have various amounts of contamination with human DNA. The relative amounts of DNA can be determined by hybridization with the probe pOV8 which hybridizes with many different filarial parasite species.

Table 3.1

<u>Filarial Parasites</u>	<u>Location</u>	<u>Source</u>
<u>Brugia malayi</u> (B.m.)	USA	TRS Laboratories
" " "	Buton, Indonesia	F. Partono
" " "	Benkulu, Indonesia	"
<u>B. timori</u> (B.t.)	Timor, Indonesia	"
<u>B. patei</u>	Africa	A. Vickery
<u>B. pahangi</u>	USA	TRS Laboratories
<u>Wuchereria bancrofti</u> (W.b.)	Indonesia	F. Partono
" "	Sri Lanka	S. Dissanayake
<u>W. kalamantani</u> (W.k.)	Indonesia	F. Partono
<u>Loa loa</u> (L.l.)	Africa	T. Nutman
<u>Mansonella ozzardi</u> (M.o.)	Venezuela	I. Petralanda
<u>Acanthocheilonea viteae</u> (D.v.)	USA	TRS Laboratories
<u>Dirofilaria immitis</u> (D.i.)	USA	"
<u>Litomosoides carinii</u> (L.c.)	USA	"
<u>Setaria</u>	Sri Lanka	S. Dissanayake
<u>Onchocerca volvulus</u> (O.v.)	Guatemala	B. Duke
<u>O. gibsoni</u> (O.g.)	Australia	L. Semprivo
<u>O. cervicalis</u> (O.c.)	-----	E. James
Nodule	-----	J. Donelson
JC 17, <u>O. gutturosa</u> (<u>O. gutt.</u>)	UK	J. Crampton
JC 25, <u>O. ochengi</u> (<u>O. och.</u>)	Mali, Africa	"
JC 31, <u>O.g.</u>	Australia	"
JC 42, <u>O.v.</u>	Mali, Africa	"
JC 56, (<u>O. gutt.</u>)	"	"
JC 64, <u>O. armillata</u> (<u>O. arm.</u>)	Sierra Leone	"
JC 69, <u>O. arm.</u>	Mali, Africa	"
JC 77, <u>O.v.</u>	Sierra Leone	"
JC 84, <u>O. arm.</u>	Sudan	"
JC 93, <u>O. gutt.</u>	Sierra Leone	"
Sav. Mali	Bamako, Mali	J. Donelson
V526 <u>O. volvulus</u>	Africa (Savanna)	M. Karam
28 "	Asubende, Ghana	"
113 "	Africa (Savanna)	"
209 "	Wahinou, Ivory Coast of	"
216 "	Africa (Intermediate	"
240 "	between Savanna and	"
	Forest)	"
	"	"
	"	"
	"	"

Table 3.2

Summary of Filarial DNA Probes Used on Filters

Species	Investigators	Probe Name	Detection System
<i>B. malayi</i>	Poole/McReynolds	BM45	biotin-phosphatase
<i>B. pahangi</i>	Poole/McReynolds	BP41	biotin-phosphatase
<i>B. malayi</i>	Williams	BM45B	biotin-peroxidase
<i>B. pahangi</i>	Williams	BP45B	biotin-peroxidase
<i>B. malayi</i>	Supali/Partono	BML	luminol
<i>B. pahangi</i>	Supali/Partono	BPL	luminol
<i>B. malayi</i>	Dissanayake/Piessens	pBM15	radioactive
<i>W. bancrofti</i>	Dissanayake/Piessens	IWb35	radioactive
<i>W. bancrofti</i>	Karunanayake	3Wb34	radioactive
<i>W. bancrofti</i>	Ragahavan	Wb1,Wb2,Wb6	luminol
<i>W. bancrofti</i>	Williams	pWb6,pWb11	luminol
<i>Loa loa</i>	Klion/Nutman	LL3	radioactive
Human	Williams	genomic	radioactive
<i>O. volvulus</i>	Harnett	C1A1-2	radioactive
<i>O. volvulus</i>	Trees/Crampton	pOV2,pOV5,pOV6	radioactive
<i>O. volvulus</i>	Unnasch	pFS-1,pSS-1BT, pOVS134	radioactive
<i>O. volvulus</i>	Meredith	pOVS134	radioactive
<i>O. volvulus</i>	Perler	pOV8,pOV26	radioactive
<i>O. volvulus</i>	Wirth/Piessens	pOV3	radioactive
<i>O. armillata</i>	Trees/Crampton	pOA1	radioactive

The species used to isolate the probes, the investigator that used them, and the probe's name are given in this table. All of the *Brugia* probes, except pBM15 are species-specific oligonucleotides from the variable region of the repeat [see text]. All of the *O. volvulus* probes, except pOV8 and pOV26, are shown by hybridization and sequence analysis to be different members of the same repeated DNA family.

Table 3.3a

Lymphatic Filariasis Map I

Lymphatic Filariasis blots labeled A - H; Numbers in () represent amounts of DNA in

ng; F. Partense - blot A; B: T. Egwang - blot D; H. Raghavan - blot E; E.

varunahashie - blot F; T. H. L. Man - blot G

	1	2	3	4	5	6	7	8	9	10	11	12
A	B.m (0.1)	B.m/B.p (0.2/0.2)	B.p (12.8)	W.b. Indo (0.05)	W.b. Sri (0.05)	W.k. (0.05)	L.I. (0.1)	H (0.1)	M.o (4.4)			
B	B.m (0.2)	B.m/B.p (0.2/0.4)	B.p (6.4)	W.b. Indo (0.1)	W.b. Sri (0.1)	W.k. (0.1)	L.I. (0.2)	H (0.2)	L.C. (12.8)			
C	B.m (0.4)	B.m/B.p (0.4/0.8)	B.p (3.2)	W.b. Indo (0.2)	W.b. Sri (0.2)	W.k. (0.2)	L.I. (0.4)	H (0.4)	D.v (12.8)			
D	B.m (0.8)	B.m/B.p (0.8/1.6)	B.p (1.6)	W.b. Indo (0.4)	W.b. Sri (0.4)	W.k. (0.4)	L.I. (0.8)	H (0.8)	D.I. (12.8)	Selaria ML3 (12.8)		
E	B.m (1.6)	B.m/B.p (1.6/3.2)	B.p (0.8)	W.b. Indo (0.8)	W.b. Sri (0.8)	W.k. (0.8)	L.I. (1.6)	H (1.6)	B.t. (12.8)	O.v (12.8)		
F	B.m (3.2)	B.m/B.p (3.2/6.4)	B.p (0.4)	W.b. Indo (1.6)	W.b. Sri (1.6)	W.k. (1.6)	L.I. (3.2)	H (3.2)	B.patei (12.8)	O.g (12.8)		
G	B.m (6.4)	B.m/B.p (6.4/12.8)	B.p (0.2)	W.b. Indo (3.2)	W.b. Sri (3.2)	W.k. (3.2)	L.I. (6.4)	H (6.4)	B.m. Suton (12.8)	O.C (12.8)		
H	B.m (12.8)	B.m/B.p (12.8/25.6)	B.p (0.1)						B.m Benkulu (6.25)	Module (12.8)		

A sample of the arrangement of the DNAs on the filters sent to the individual investigators. The letters or numbers represent the species or isolate of the individual parasite samples (table 3.1). The number of nanograms of DNA in the sample is shown in parentheses. The DNA quantitation was determined by gel electrophoresis with known DNA standards.

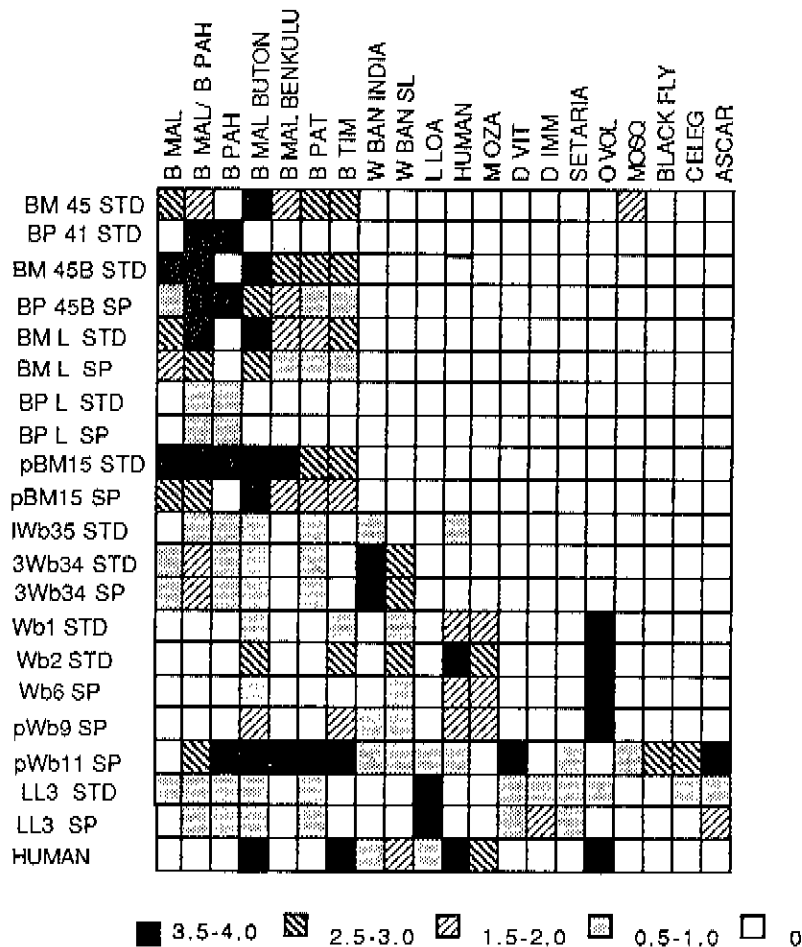
Table 3.3b

ONCHOCERCA MAP I

Onchocerca blots labeled 1-4, Numbers in () represent amounts of DNA in ng.
Missing samples 173 & 404, J. Crampton - blots 1, 2, 3, 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	Human (102.4)	O.v. (102.4)	/	Ascaris (102.4)	Brugia malayi (25.6)	Nodule (85.6)	/	JC 31 (22.6)	V526 (102.4)	305 (<1)	161 (7.0)	11 (239.0)
B	Human (51.2)	O.v. (51.2)	/	C.elegans (102.4)	D.i. (102.4)	O.c. (102.4)	/	JC 42 (<2)	Sav. mali. (82.7)	306 (6.1)	170 (173.0)	22 (6.7)
C	Human (25.6)	O.v. (25.6)	/	Black Fly (102.4)	D.v. (102.4)	O.g. (102.4)	/	JC 56 (<2)	/	307 (<1)	209 (<1)	28 (42.0)
D	Human (12.8)	O.v. (12.8)	/	Mosquito (5.5)	L.c. (102.4)	/	/	JC 64 (<2)	/	400 (<1)	216 (<1)	44 (102.4)
E	Human (6.4)	O.v. (6.4)	/	M13 (12.8)	L.L. (25.6)	/	/	JC 69 (5.0)	/	401 (15.9)	220 (14.3)	64 (24.6)
F	Human (3.2)	O.v. (3.2)	/	pBr322 (12.8)	M.o. (4.4)	/	/	JC 77 (2.6)	/	402 (2.4)	240 (5.48)	92 (182.0)
G	Human (1.6)	O.v. (1.6)	/	Setaria (12.8)	/	/	/	JC 84 (5.5)	JC 17 (45.0)	403 (<1)	301 (19.0)	113 (15.5)
H	Human (0.8)	O.v. (0.8)	/	/	/	/	/	JC 93 (20.8)	JC 25 (12.5)	405 (10.2)	304 (<1)	115 (10.2)

Table 3.4 Lymphatic DNA Probes



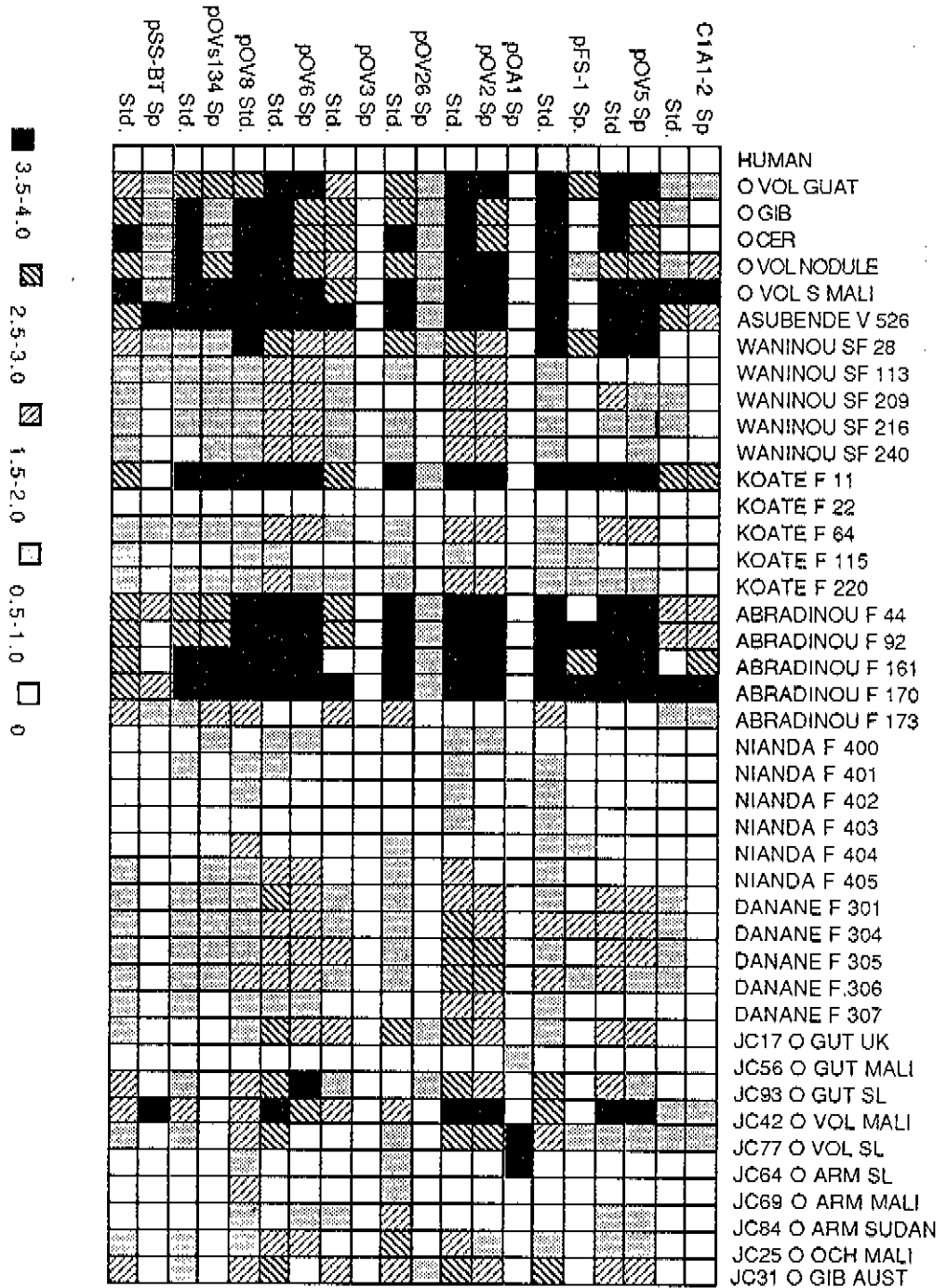
Each investigator was asked to rate the intensity of hybridization, 4 being most intense and 0 for no hybridization. The numbers are represented as shades. The following DNAs did not hybridize and are therefore not shown in the tables.

For the lymphatic DNA probes: *Wuchereria kalamantani*, *Litomosoides carinii*, *O. volvulus* and *O. guttersosa*.

For the Onchocerca probes: *Ascaris*, *C. elegans*, blackfly, mosquito, *B. malayi*, *A. viteae*, *D. immitis*, *L. carinii*, *Loa loa*, *M. ozzardi*, and *Setaria*.

Only the cross-reactive probe pOV8 hybridized to *Ascaris*, *C. elegans*, mosquito, *B. malayi*, *D. immitis*, *A. viteae*, *L. carinii*, and *Setaria* DNA.

Table 3.4 Onchocerca DNA Probes



4. RECOMMENDATIONS

The recommendations from the workshop are divided into two sections: lymphatic filariasis and onchocerciasis. Recommendations for the two types of parasites will be detailed separately.

4.1 Recommendations: DNA Probes for Lymphatic Filarial Parasites

Four major uses for DNA probes for lymphatic filarial parasites were identified. For each use, specific recommendations for basic laboratory research and for field applications are listed.

4.1.1. Use of DNA probes for screening vector populations.

Since excellent DNA probes exist for detecting B. malayi and B. pahangi, it was agreed that these probes will prove to be very useful for mass screening of vector populations. The conditions under which these probes are both sensitive and species-specific have been clearly defined. The following recommendations were made for further research:

4.1.1.1. The use of non-radioactive probes to detect DNA from L3 larvae in mosquitoes has been demonstrated in the laboratory. These results need to be verified in the laboratory and then field tested in several different locations to validate the method. Such "cold" probes will be essential for the application of DNA probes in the field.

4.1.1.2. Since a stage-specific DNA probe is not yet available for detecting L3 larvae, a method for estimating the number of L3 larvae using the current DNA probes should be developed. A study that examines the distribution of L1, L2, and L3 larvae in the bodies of infected mosquitoes should be undertaken. Information gained from such a study will provide a correction factor that will allow the current DNA probes to be used to estimate the number of L3 larvae in infective mosquitos. This method should be compared to dissection in a field study to determine its usefulness for epidemiology.

4.1.1.3. A simple method must be developed for storing the mosquitoes at ambient temperature so they can be mailed from remote regions to a central laboratory for testing. DNA assays are most likely to provide reliable results if they are conducted in central or regional laboratories with good quality control.

4.1.1.4. The use of the polymerase chain reaction (PCR) to detect very low levels of transmission in mosquito populations needs to be studied. The use of such a sensitive method will prove especially useful in monitoring the effectiveness of control programs. This method will have to be worked out first in the laboratory and then tested in the field, again using "cold" probes.

4.1.2. Use of DNA probes for screening human blood samples.

The same DNA probes discussed above have also been used in a parasite detection assay for screening human blood samples (Poole, C.B. and Williams, S.A., 1990, Molecular and Biochemical Parasitology, 40, 129-136). This assay has been tested very successfully in one field trial in Indonesia using radioactive DNA probes (Williams, S.A., McReynolds, L.A., and Partono, F., manuscript in preparation). It was agreed by the workshop participants that this method should prove very useful for screening human and animal populations. The following recommendations were made for further research.

4.1.2.1. The use of non-radioactive DNA probes to detect microfilarial DNA in human blood samples has been demonstrated under laboratory conditions. Field testing in several different locations is now required to validate the method. The new method must be compared to the traditional stained blood smear method to assess the relative sensitivity, species-specificity, reliability, accuracy, and cost. Again, the use of "cold" probes will be essential for acceptance of the DNA probes as a useful diagnostic tool.

4.1.2.2. A simple method has been developed for collecting blood samples in a solution of EDTA. This solution preserves the parasite DNA in blood for at least one month at 37° C. Samples can be collected in 1.0 or 1.5 ml plastic tubes containing EDTA and can then be sent to a central or regional testing facility. This method needs to be evaluated in field studies in a variety of locations.

4.1.3. The Possible Use of DNA probes in hospital or clinical studies.

The DNA probes for Brugian filariasis may also prove useful in the examination of blood and tissue samples from hospital or other clinical patients. Research should be undertaken to assess the feasibility and advisability of such applications. The polymerase chain reaction (PCR) technique is appropriate for studies of this type because of the great sensitivity provided at a relatively modest cost per assay. The following specific recommendations were proposed for further research.

4.1.3.1. A clinical study needs to be undertaken which uses a combination of PCR and non-radioactive DNA probes to examine patients for the presence of low numbers of microfilariae in the blood. For example, individuals showing acute pathology often show no microfilariae when tested by traditional means. An extremely sensitive assay using PCR could be used to test for very low levels of microfilaremia.

4.1.3.2. A clinical study should be undertaken using this method to examine tissue biopsies for the presence of

microfilariae. For example, lung biopsies from patients with tropical pulmonary eosinophilia could be examined.

4.1.4. Use of DNA probes and DNA sequence analysis to correlate different strains of filaria with different clinical disease manifestations.

A large DNA sequence data base already exists comparing Brugia parasites from different geographic locations within Indonesia. The Brugia malayi isolates include parasites considered to be anthropophilic, zoophilic, periodic, and sub-periodic. With these data, it may be possible to design DNA probes specific for various strains or sub-species of filarial parasites. It was agreed that such DNA sequence data and DNA probes will prove valuable in assessing the relationship between different strains of filaria and different clinical manifestations of disease. For example, for Wuchereria bancrofti, the severity of disease is seen to increase moving from Jakarta in the west to Irian Jaya in the east. It is also recognized that urban W. bancrofti seen in Jakarta is different than the rural forms found elsewhere in Indonesia. Could these differences be due to genetic differences in the parasite? That is, do different strains of W. bancrofti exist that show significant differences in pathology or other biological properties? Another question that may be addressed with the same type of data would involve looking at parasites from individuals showing different forms of the disease. Do individuals with tropical pulmonary eosinophilia possess Brugia of a different type than those individuals with elephantiasis or those infected but showing no pathology? These are questions that can be addressed using DNA sequence data and DNA probes. The recommendations for further research are as follows.

4.1.4.1. In addition to those filariae that have already been collected and analyzed, additional specimens must be obtained from various locations showing important biological differences (for example, W. bancrofti from Jakarta and Irian Jaya). Specimens must also be collected from patients exhibiting different clinical manifestations of the disease (for example, patients with elephantiasis and others with TPE). DNA sequence data must be obtained from these various isolates and examined for consistent differences. If such differences are found, then the design of DNA probes to detect these different types can be undertaken.

4.1.4.2. Repeat DNA sequences may not be the most sensitive indicators of strain differences. Therefore, other DNA sequences should be cloned and examined. Intervening sequences in coding genes may prove superior in indicating diagnostic differences between closely related strains.

4.1.4.3. Further work needs to be done to improve the DNA probes being developed for W. bancrofti. These new probes,

once proven sensitive and species-specific in laboratory tests, should be validated in a number of field studies undertaken in a variety of different geographical locations. Again, the use of non-radioactive probes will prove crucial in the development of parasite detection assays with new probes.

4.2 Recommendations: DNA Probes for Parasites Causing Onchocerciasis

Four major uses were identified for the strain and species-specific DNA probes for Onchocerca volvulus. The following recommendations address research activities designed to improve the utility of the Onchocerca DNA probes in achieving these objectives.

- 4.2.1. Use of DNA probes to monitor breakthrough transmission in both vector and drug control programs, and to identify the source of the breakthrough parasites.
- 4.2.2. Use of DNA probes to monitor geographic shifts in parasite and vector populations that may relate to disease control.
- 4.2.3. Use of DNA probes to monitor seasonal shifts in zoophily which may, in turn, affect transmission rates of O. volvulus.

Five specific recommendations were made for research required to achieve the above three uses for Onchocerca DNA probes:

4.2.3.1. Since DNA probes will be most reliable when used in central or regional laboratory facilities, it will be necessary to devise methods which will enable the collection and transport of L3 larvae and infected flies that maintain the integrity of the parasite DNA. Thus, research on the collection, storage, and transport of infective larvae and infected flies is an important research priority. The methods used may differ depending upon the type of study being conducted (for example, examination of flies in bulk versus individual flies, or dissected versus intact flies).

4.2.3.2. Further research needs to be done on methods which will efficiently and reliably disrupt Onchocerca L3 larvae. For the DNA probes to prove useful in a parasite detection assay, a simple method to release DNA from the L3 larvae is absolutely essential.

4.2.3.3. Since the first two recommendations will involve the need for large numbers of L3 larvae for research purposes, it is recommended that TDR/FIL should make this material more readily available, perhaps by sponsorship on a contract basis.

4.2.3.4. The sensitivity of the Onchocerca DNA probes must be increased without a loss in the specificity. This increase in sensitivity must be accomplished using non-radioactive

methods of detection.

4.2.3.5. To confirm the increase in sensitivity without loss of specificity, it is recommended that a bank of material be established that consists of L3 larvae, infected flies, and genomic DNA from different Onchocerca species and isolates from different geographic regions. This bank should be created and maintained by TDR/FIL on a contract basis. Particular attention in developing the bank should be paid to Onchocerca isolates from African ungulates and to O. volvulus from Latin America. Investigators supported by TDR/FIL should be encouraged to provide material to this bank.

4.2.4. To investigate the possible advantages of using DNA probes to detect prepatent and occult infections, and to monitor microfilaria levels in lightly infected and ivermectin treated individuals.

4.2.4.1. It is recommended that methods be developed to release DNA from microfilariae found in the skin. This is likely to be a difficult problem since previous efforts in this area have not been successful.

4.2.4.2. It is recommended that DNA probes for diagnosis be developed in conjunction with immunological and classical parasitological methods of diagnosis. In this way, the advantages and disadvantages of DNA probe based diagnosis will be easy to assess.

4.3. Final Recommendations on DNA Probes for Filarial Parasites

4.3.1. New developments in DNA probes for screening human or insect populations must concentrate on the development of non-radioactive probes. It was concluded that radioactive probes will not be very useful in developing countries.

4.3.2. It is imperative that simple methods be developed for collecting, preparing, and shipping specimens from the field to a central or regional laboratory for DNA testing. These methods should enable samples to be sent by mail with no refrigeration required. Collecting blood samples on filter paper or collecting mosquitoes in plastic envelopes are examples of methods that should be explored.

4.3.3. The technology for producing and labeling the DNA probes needs to be transferred to the countries where the diseases are endemic. Such an arrangement will lead to decreased costs of screening programs.

4.3.4. The development of the new DNA probe technology should be carried out, not at the expense of classical parasitological techniques, but in conjunction with them. Excellent morphological based methods of diagnosis (for example, stained blood films) will be needed to validate the new techniques.

Such validation should be done in a variety of field studies conducted in numerous locations involving different forms of each parasite.

- 4.3.5. The DNA probes being developed for W. bancrofti are promising but need further laboratory based research to work out precise hybridization conditions for sensitivity and specificity. Once these have been determined, the probes must be tested using non-radioactive detection in a variety of field tests.
- 4.3.6. The new technique of target amplifications, the polymerase chain reaction, should be fully explored as the most sensitive method for detecting low levels of microfilariae in human fluids and tissues, and for detecting low levels of infection in insect vector populations. Whether or not such methods are feasible in endemic regions needs to be fully assessed.

APPENDIX I. SYMPOSIUM ON FILARIASIS AND ONCHOCERCIASIS - 18 Dec. 1989

The opening ceremony was presided over by Prof. Dr. A.A. Loedin, Assistant Minister of State for Research and Technology, Republic of Indonesia; and the proceedings were opened by Dr. Sumarmo Poorwo Soedarmo, Head of the Indonesian National Institute of Health Research and Development. Prof. Dr. Bintari Rukmono, Head of the Department of Parasitology, University of Indonesia, and chair of the Organizing Committee, welcomed all of the participants and observers to the Symposium and Workshop. Dr. Rana K. Jung, as the W H O. representative to Indonesia, welcomed all of the participants on behalf of the W H O.

Dr. Larry A. McReynolds of New England Biolabs, Inc. then gave the Symposium and Workshop overview. He explained that the purpose of the meeting was to bring together scientists developing DNA probes with those working on filariasis in endemic regions to determine the most useful applications of DNA probes and to chart the course of future research needed to make field use of DNA probes a reality. He explained that coded filters with DNA samples of many species of filaria were sent to about 12 investigators who have developed DNA probes, so that all of the probes could be tested for species-specificity and sensitivity. The results of this survey were presented later in the meeting.

Dr. C.P. Ramachandran then presented an overview of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). He explained that WHO and TDR's great interest in this meeting is due to the great need for improved diagnostics for filariasis in endemic regions. He then gave an excellent summary of TDR's goals in sponsoring research and training on the prevention, diagnosis, and treatment of selected tropical diseases including filariasis. He stressed that new tools for prevention, diagnosis, or treatment must be effective, appropriate, and affordable for use in endemic nations. He gave several examples of TDR sponsored research conducted in Indonesia and stressed his desire for these close ties to continue in the future. Dr. Ramachandran explained that TDR's current and future focus will be on product development and transformation of new scientific technologies into useful disease control tools. Sponsorship of this meeting with New England Biolabs is an example of this focus. New tools for diagnosing filariasis must be developed, because current methods cannot identify prepatent infections or amicrofilaremic individuals. TDR is actively supporting development of DNA probes and immunological tools to rectify this situation. He expressed his desire that this meeting of experts in molecular biology would help to understand and solve some of the problems of filariasis diagnosis.

Dr. Sumarmo P. Soedarmo, Head of the National Institute of Health Research and Development of Indonesia, then opened the proceedings with an address on filariasis research and biotechnology in the Republic of Indonesia. He acknowledged the contributions that advances in biotechnology, such as DNA probes,

are likely to make in solving important health problems. He stressed that introduction of a new technology in an endemic country must be done in the context of the existing knowledge base. The strategy for moving the new technology into the health care system, the evaluation of the efficiency and safety of the new technology, and education in the use of the new technology are all important points that must be considered. Wishing the participants success, Dr. Soedarmo opened the proceedings.

SYMPOSIUM ON FILARIASIS AND ONCHOCERCIASIS - SUMMARY OF DISCUSSIONS

Filariasis: The Parasite and Its Host

The first scientific sessions of the Symposium on Filariasis and Onchocerciasis was chaired by Prof. Dr. Bintari Rukmono and co-chaired by Dr. Kurt Sorensen.

Lymphatic Filariasis: Clinical Diagnosis and Treatment

Dr. Felix Partono

Lymphatic filariasis is caused by the lymphatic dwelling filarial parasites, Wuchereria bancrofti, Brugia malayi, and Brugia timori. The females produce microfilariae which circulate in the peripheral blood with either a nocturnal or diurnal periodicity. Transmission occurs through an infective bite of a vector mosquito of the genus Culex, Aedes, Anopheles or Taeniarhynchus (Mansonia).

The disease is prevalent in the slum or rural areas of many tropical and subtropical countries, predominantly affecting the poorer sector of the community. The acute stage is characterized by episodic inflammation of the lymphatic glands and vessels, resulting in loss of working hours, followed by disfiguring lesions of the extremities and genital organs one or more decades later.

There are currently 90 million people infected with the parasites throughout the world, and two-thirds of them live in China, India, and Indonesia. More than 20 million Indonesians are at risk of acquiring the parasites and between 3 and 4 million people are infected.

Transmission of the disease depends on the presence of large numbers of vector mosquitos and an adequate human or animal reservoir with microfilaremia. Not every microfilaremic person is a good source of infection. An infected blood meal containing many microfilariae is prone to kill the mosquito, and with a lower count the mosquito is not likely to pick up the infection. Repeated infections over a long period are necessary for the development of microfilaremia or clinical filariasis among natives. Visitors making brief stops of 1 to 2 weeks in endemic areas are probably

not at risk, but migrants moving from non-endemic to endemic filarial areas are more prone to develop clinical filariasis within 3 to 6 months.

The disease should be divided into two distinct clinical syndromes: 1) lymphatic filariasis, caused by the adult or developing adult worms, producing episodic inflammation of the lymphatics and obstructive lymphatic lesions; and 2) occult filariasis, including tropical pulmonary eosinophilia, due to a hyper-immunological response of the host against the microfilariae.

Occult filariasis occurs occasionally in Indonesia, India, South America, and South East Asia. The disease is characterized by lymphadenopathy and/or bronchial asthma, which can be effectively treated with diethylcarbamazine (DEC). Laboratory findings include hypereosinophilia, increased filarial specific serum IgE, and characteristic histological lesions (Meyers Kouwenaar bodies) in the lymph nodes, lungs, spleen, and liver. Microfilariae are usually absent in the blood.

The course of lymphatic filariasis among natives starts with the prepatent period of 3 to 7 months, followed by asymptomatic microfilaremia, acute and then chronic filariasis. Among migrants from non-endemic filarial areas, the disease may start with adenolymphangitis within 2 to 3 months after arrival, and chronic lesions may develop within 6 to 12 months. Microfilaremia develops much later.

In brugian filariasis, the clinical features are episodic lymphadenitis, retrograde lymphangitis, abscesses, ulcers, and scarring in the acute stage, followed by elephantiasis of the limb below the knee, and sometimes that of the arm below the elbow.

In bancroftian filariasis, episodic inflammation of the lymph vessels or glands predominantly affects the male genitalia, producing funiculitis, epididymitis, or orchitis. Adenolymphangitis affecting the lymphatics of the extremities is less common. In the chronic stage, hydrocoele is the most common feature. Elephantiasis affects the whole limbs, the whole arms, the scrotum, the vulva or the breasts. Chyluria usually occurs intermittently and without symptoms, but some patients complain of fatigue and weight loss.

Clinical diagnosis of lymphatic filariasis is firmly established when a history of clinical manifestations of acute or chronic filariasis are associated with demonstrable microfilariae in blood, hydrocoele fluid, or urine. When microfilariae are absent, the following criteria are indicative of filariasis. Patients from endemic areas of filariasis with clinical history or signs of episodic lymphadenitis, retrograde lymphangitis, or orchitis, or with chronic obstructive lesions, should be suspected of having a filarial infection, even when microfilariae are absent in their night blood samples.

To date, DEC remains the drug of choice for the treatment and control of lymphatic filariasis. The drug is micro- and macrofilaricidal. For bancroftian filariasis, 6 mg DEC per kg for 12 doses is the WHO recommended schedule. For brugian filariasis, 3 to 6 mg DEC per kg, up to a total dose of 18-72 mg per kg have been used by various Asian countries in their national control programmes. The drug can be given daily, weekly, monthly, or even yearly. Several courses of DEC are needed for an optimal result. For individual treatment, a daily schedule is more convenient. Side reactions include fever, headache, other constitutional symptoms or local symptoms such as adenolymphangitis, or transient lymphoedema. These symptoms usually occur 1 to 2 days after the first dose, last 2 to 5 days, and are usually mild. A weekly schedule is more acceptable for community treatment. To avoid side reactions, DEC can be distributed in low doses (50 to 100 mg weekly) by village volunteers for a duration of 8 to 18 months. Alternatively, DEC can be given in table salt, medicated with 0.1-0.4% DEC for a period of 6 to 18 months. It should be highlighted that lymphatic filariasis can be systematically eradicated by distributing DEC alone, without controlling the vector. The ultimate aim of the Indonesian Filariasis Control Programmes is to create a community free of filariasis.

Onchocerciasis--Dr. Bruce M. Greene

Onchocerciasis is the most socio-economically devastating disease known to humans. 20 million individuals in Africa and the Americas are infected with Onchocerca volvulus. It is the fourth leading cause of blindness in the world today. Individuals who are blind due to O. volvulus show a four-fold increase in mortality. Major foci of onchocerciasis are in equatorial Africa, Guatemala, southern Mexico and Brazil. There is currently no practical cure for this disease. The vectors of O. volvulus are all black flies belonging to the genus Simulium. These vectors deposit their eggs in fast-moving rivers and streams; thus the disease is termed African River Blindness.

Adult Onchocerca form nodules in the human body that typically contain two to three females and one male. Adults are large with females being 40 - 60 cm in length. The adult females shed microfilariae which live in the dermis just below the epidermis. The standard diagnosis involves placing tissue from a skin biopsy (skin snip) in saline for a few hours and then examining the solution for microfilariae using an inverted microscope.

Besides nodules, other clinical manifestations of the disease include various dermatological manifestations and ocular lesions. In Africa, the nodules are most often found near the pelvis and other body locations below the neck. In America, most nodules are seen on the head and neck. Dermatological manifestations include dermatitis, atrophy, pigmentary changes and intense itching. Ocular lesions can include damage to the retina, cornea, lens and

elsewhere. Hanging groin syndrome results from chronic lymph node inflammation, scarring, and eventual blockage followed by collection of fluid around these nodes. This lymph node involvement is not due to adults living in the lymphatics.

The disease blinds adults who should be in their most economically productive years. The increased mortality associated with blindness is not understood. Disease manifestations, at least in part, are likely to be immune mediated. For example individuals with limbitis show a higher cell mediated immune response than those with no limbitis. This is an important point that must be considered in drug and vaccine development. Great care must be taken that such measures do not aggravate or increase the severity of the disease.

Vectors of Onchocerciasis--Dr. Stefanie Meredith

Blackflies of the genus Simulium are the vectors of onchocerciasis in both Africa and America. Control of the disease in West Africa (Onchocerciasis Control Programme) has focused solely on the vector. Eggs of the vector are laid in fast-running water. Eggs, larvae and pupae all develop in the water affixed to rocks and other solid supports.

The most important vectors in Central and West Africa belong to the S. damnosum complex of species. These vectors have been studied intensively since the establishment of the Onchocerciasis Control Programme in West Africa in 1974. In 1969, only one species of S. damnosum was known. Now, more than 20 species and forms have been described. These various species have been defined by examination of the polytene chromosomes found in the larvae. Inversions have been the most important cytogenetic features used in defining the various species and forms.

Savannah regions of West Africa are the most severely affected by this disease. In these regions, fast-running water may only flow three to four months during the year. In this region there are seven or eight different S. damnosum species. All of these species can serve as vectors for Onchocerca, although they vary greatly in their ability to transmit the parasite. One crucial factor is the life span of the vector. If it is too short, very few parasites will develop to the L3 stage and be transmitted. Much has been learned over the past 20 years about the vector, and now molecular biology is providing more information about the parasite. Research will now be able to focus on vector/parasite interaction. Such studies will be vital for the design of optimal control programs. The Onchocerciasis Control Programme has been in existence for only 15 years and has focused solely on vector control. Nevertheless, transmission has been reduced to very low levels in an area that was formerly very high transmission.

Diagnosis of Filariasis

The second session of the Symposium on Filariasis and Onchocerciasis was chaired by Dr. Sri Oemijati and co-chaired by Drs. Hoedojo.

Parasitic Diagnosis and Vector Incrimination in Lymphatic Filariasis--Drs. Purnomo

The life cycle and periodicity of lymphatic filarial parasites was reviewed. For clinical and practical purposes, the microfilaria is the most accessible stage and the easiest to identify. Microfilariae can be found in the blood, hydrocoele fluid, urine and tissues. The time of blood collection should be as close as possible to the peak of microfilarial periodicity for the species and strains concerned. Blood can be collected by digital puncture or venepuncture. The detailed morphology of Brugia malayi, Brugia pahangi and Brugia timori microfilariae was described and illustrated. Methods for obtaining accurate counts of microfilariae using blood smears and membrane filtration were reviewed.

Adult worms can be obtained from biopsy tissue (post-operative on lymph nodes). Adult males are about 2 cm in length, while females are about 4 cm in length. Species identification based on adult worms depends on a knowledge of spicule structure in adult males. Adult females can be used if histological cross-sections enable the examination of microfilariae.

The easiest method for incriminating potential vectors of lymphatic filariasis is to collect blood-fed mosquitoes in bed nets of microfilaremic patients. These mosquitoes can then be reared in the laboratory and examined for L3 stage larvae. As many specimens of the incriminated vector should be collected as possible. All species and strains of mosquitoes which are shown to be capable of supporting the development of the ingested microfilariae to the infective stage, must be considered potential vectors.

Diagnosis of Onchocerciasis--Dr. Thomas Nutman

Any new diagnostic assay should meet the following requirements: it should be sensitive, discriminating, quantifiable, reproducible, simple, inexpensive, field compatible, and automatable (in order to screen large populations).

A variety of methods exist for diagnosing onchocerciasis based on classic parasitological techniques. Examination of skin snips incubated in saline for microfilariae is the standard technique. The sensitivity of this method can be improved by digesting the snipped tissue in collagenase. The part of the body from which the

skin snips are obtained is a very important factor in determining the sensitivity of this assay. The best location for skin snips depends on the region of the world in which the study is being done. Increasing the number of snips from two to four to six greatly increases the sensitivity of the assay. More than six snips per individual gives very little increase in sensitivity.

Another parasitological technique for diagnosing onchocerciasis is to do nodulectomies to look for adult worms. Again the part of the body examined will depend on the region of the world in which the work is being done. It should be remembered that nodules can be caused by a variety of problems that have nothing to do with filarial worms. About six per cent of nodules removed in endemic regions are not due to Onchocerca. The procedure is relatively insensitive for diagnosis, it is invasive, and it requires highly trained personnel. A non-invasive procedure for identifying nodules in the field is to use a portable ultrasound device. Unfortunately, this does not result in a definitive diagnosis.

Provocative tests can also be used to diagnose onchocerciasis. The Mazzotti test involves giving 50 mg of DEC in order to elicit symptoms that allow a diagnosis to be made. Infected individuals will show a variety of responses to the drug, especially dermatological manifestations. However, severe side effects make this test undesirable. A more acceptable provocative test involves a topical application of DEC. Even with this method, severe reactions are not uncommon. Since provocative tests are no more sensitive than skin snips, and since they can give false positive results and often give severe side reactions, they are not recommended for diagnosis.

Immunological tests of various kinds have been developed for use in diagnosis of onchocerciasis. A large number of early serodiagnostic tests, some using immunofluorescence, lacked species-specificity and were relatively insensitive. ELISA's and RIA's improved the sensitivity of these tests, but not their specificity. Detection of O. volvulus circulating antigen as developed by A. Capron and coworkers had similar problems. These parasites are skin-dwelling, so there is little parasite antigen in the circulation. The test proved to be relatively insensitive and gave false positive results. A breakthrough came when N. Weiss and coworkers used fractionated low molecular weight antigen of O. volvulus. This test gave improved specificity and sensitivity when compared to unfractionated antigen. The use of recombinant cloned antigens may give even better specificity and sensitivity. The purified protein can be used to develop an ELISA assay that can detect antibodies in even prepatent and very light infections.

Molecular biology may prove useful in Onchocerca diagnosis. Recombinant DNA techniques can be used to clone genes coding for parasite antigens that can then be used in ELISA assays. Cloned DNA probes may prove useful for identifying microfilariae in tissues, blood or other bodily fluids.

New Horizons for Identification and Treatment

The third scientific session of the Symposium on Filariasis and Onchocerciasis was chaired by Dr. H. S. Sidhu and co-chaired by Dr. L. Kurniawan.

New Diagnostic Methods--DNA Probes--Dr. Steven A. Williams

A variety of new diagnostic tools are being developed which may prove useful in gathering epidemiological data in large-scale field studies. Any new diagnostic technique must meet the following three criteria: 1) the assay must be at least as sensitive as standard parasitological techniques, 2) the assay must be exquisitely species-specific, and 3) the assay must be applicable for use in endemic nations. Some of the new methods are being developed to detect microfilariae in blood samples, while others are designed to detect L3 larvae in mosquito samples. These methods include various immunological screening techniques such as Dr. Gary Weil's antigen assay for screening blood samples and Carlow and Philipp's monoclonal antibody for detecting L3s in mosquitoes. DNA probes have also been developed for detecting various species of filariae including Brugia malayi, Brugia pahangi, Wuchereria bancrofti, Onchocerca volvulus and Loa loa. The ultimate goal in developing these DNA probes is to be able to use them in practical assays to detect filariae in blood and mosquitoes.

DNA probes are pieces of DNA derived from the type of parasite one wishes to detect. These pieces of DNA are cloned into plasmid or bacteriophage vectors using standard recombinant DNA techniques. Clones of value are those that can detect DNA from the parasite of interest with great sensitivity and specificity. Ideally, the DNA probe should be sensitive enough to detect a single worm in a blood sample or in a mosquito. The most sensitive DNA probes are those based on highly repeated DNA sequences. The DNA probe must also be species-specific, that is, the DNA probe must not detect DNA from any other species of filariae, nor should it detect DNA from mosquitoes or the vertebrate host. The sequence of bases in the DNA probe are complementary to the sequence of bases in the repeated DNA of the parasite. If the DNA probe is labeled with a radioactive or non-radioactive reporter molecule, the presence or absence of the target DNA (the parasite DNA) can be determined.

For example, the DNA probes we have developed for detecting Brugia malayi are based on a repeated DNA family (the Hha I repeat DNA family) that makes up 10-12% of the Brugia genome. Based on DNA sequence data collected from more than 60 cloned B. malayi and B. pahangi repeats, species-specific oligonucleotide probes have been devised. These DNA probes can be labeled radioactively with ³²P or ³⁵S, or they can be labeled non-radioactively with biotin or the enzyme horseradish peroxidase. The assay we have developed for detecting these parasites in blood samples is really quite simple. The blood samples are filtered through a nitrocellulose membrane.

filter and then treated with sodium hydroxide. The filters are baked and then hybridized with the labeled DNA probe. Excess probe is washed from the filters, and the probe that is bound to the membrane via the target DNA is detected by the appropriate assay. We have developed a similar assay for detecting L3 larvae in infected mosquitoes.

Assays based on non-radioactive means of detection have obvious advantages over radioactive assays. Non-radioactive materials are often easier to obtain, are stable for long periods of time, do not require special training or facilities, and do not require any special disposal procedures. One problem with non-radioactive probes is that they are usually much less sensitive than radioactive probes. Two recent advances have virtually eliminated this problem of sensitivity. First, various new methods of non-radioactive labeling and detection of probe DNA have greatly improved sensitivity over the old methods. These methods include improved procedures for labeling DNA molecules with biotin. Another new method that shows great promise involves labeling DNA with the enzyme horseradish peroxidase, and then detecting that probe using chemiluminescent molecules. Second, sensitivity can be improved by amplifying the amount of target DNA that is to be detected. This method uses the polymerase chain reaction and can amplify the target DNA so that a single microfilaria can be detected with even the least sensitive non-radioactive methods. We have used the PCR method successfully on both blood and mosquito samples using non-radioactive assays.

I believe the future of DNA probe use in the field is dependent on how "user-friendly" we can make the parasite detection assays. The design of simple and efficient assays is probably even more important, and certainly more difficult, than the development of the DNA probes themselves. Once a method is available that uses extremely sensitive and species-specific DNA hybridization probes in an assay that is non-radioactive, quantitative, fast, inexpensive and easy to learn; we hope that kits will be produced for use by epidemiologists, clinicians and other health care personnel working in endemic regions.

A New Drug for Onchocerciasis--Ivermectin--Dr. Bruce Greene

Side effects due to treatment with diethylcarbamazine (DEC) can cause patients with onchocerciasis to become very sick. For example, ocular disease can be aggravated by treatment with the drug. For these reasons, patients infected with Onchocerca volvulus have rejected treatment with DEC. A new alternative drug is ivermectin, a drug purported to have fewer side effects than DEC.

A study was undertaken to assess the side reactions and effectiveness of a single dose of ivermectin compared to a standard course of DEC treatment. This study, done in Africa, was a double-blind, placebo control study. Ten individuals received a single

dose (150 ug/kg) ivermectin, 10 individuals received a standard course of DEC, and 10 individuals received the placebo. Each individual was examined once per day and given a clinical reaction score based on clinical manifestations such as the severity of skin rash, fever, etc. The DEC treated group showed significantly higher clinical reaction scores than the ivermectin treated group. The ivermectin group did not show a significantly higher score than the placebo group. Data also showed that ivermectin was very effective in reducing the skin microfilaria counts. However, if individuals were not retreated, the skin microfilaria counts rose to appreciable levels by 12 months post-treatment. The single ivermectin dose was shown to be effective in reducing ocular disease for as long as 24 months.

In another study, the effect of ivermectin treatment on severe onchodermatitis was observed in 21 patients. In most patients, a significant improvement was seen at three months post-treatment. At six months post-treatment, a return of dermatological problems was observed. In a long-term community study involving 14,000 people in 73 villages, ivermectin was determined to be safe and effective. Community acceptance of the treatment was good. Ivermectin is clearly the drug of choice for treating O. volvulus infections. Another study revealed the possibility that ivermectin reduces transmission of the disease, but this must be investigated further.

The second half of the third scientific session of the Symposium on Filariasis and Onchocerciasis was chaired by Dr. C. P. Ramachandran and co-chaired by Dr. I. Ilyas.

A New Drug for Lymphatic Filariasis--Ivermectin
Dr. Kumaraswami

Two studies have been conducted in India to examine the effectiveness and safety of ivermectin in treating individuals infected with Wuchereria bancrofti. In the first study, 40 male patients with asymptomatic microfilaremia were divided into four dosage groups of 10 patients each. The four groups received ivermectin doses of 200, 100, 50 or 25 ug of ivermectin per kilogram of body weight. In all four groups the microfilaria levels dropped to essentially zero by day five post-treatment (although the drop was somewhat slower in the 25 ug/kg group). By day 12 post-treatment, there were no patients with microfilariae. By three months post-treatment, individuals were showing microfilaria levels at 10-20% of the pre-treatment levels. Serious side effects were not observed in this study.

In the second study, the effectiveness and side effects of DEC and ivermectin were directly compared. This study was a placebo control, double-blind study involving four different treatment

groups. The first group received a single low dose of ivermectin (20 ug/kg) on day one. The second group received a single high dose of ivermectin (120 ug/kg) on day one. The third group received a standard 12 day course of DEC and the fourth group was the placebo group. After five days, the code was broken for the placebo group and these individuals were reallocated to one of the other three treatment groups. The short-term assessment showed that ivermectin cleared the microfilariae from the blood faster than DEC, but that microfilariae counts were close to zero in all three drug treated groups by day 12. (Note: two of the DEC treated patients still showed very low levels of microfilariae on day 12). The long-term assessment showed that at one month post-treatment, microfilaria levels were starting to rise. By three months post-treatment, the microfilariae levels were higher in the ivermectin treated groups than in the DEC treated group. By six months post-treatment the ivermectin treated groups showed significantly higher levels of microfilariae than the DEC group. The low dose ivermectin group showed about the same level of clinical side effects as the DEC treated group. The high dose ivermectin group showed a significantly greater level of clinical side effects. The level of side reaction was found to correlate with the worm burden in the patient (based on pre-treatment levels).

Ivermectin treatment of Wuchereria bancrofti infected individuals was shown to reduce microfilaria levels faster than DEC. However, the effects of DEC in reducing microfilaria levels were found to be longer lasting. The side reactions of ivermectin in the low dose group were found to be comparable to that of DEC. Since ivermectin requires only a single dose treatment, community acceptance of this drug may be better than for DEC.

A New Drug for Brugian Filariasis--Ivermectin
Dr. Felix Partono

In this study, the efficacy of ivermectin in treating brugian filariasis was examined. Forty asymptomatic microfilaria patients from Sumatra were divided into four treatment groups and received single ivermectin doses of 20, 50, 100 or 200 ug/kg. These patients were brought to Jakarta, where they spent 16 days in the hospital following treatment so they could be carefully observed. A variety of side reactions was observed. For example, 25 of the 40 had fevers, 11 of 40 complained of headaches, and some experienced a significant decrease in blood pressure. Mean microfilaria counts were shown to decrease in all four treatment groups, although about 8% of pre-treatment values was the best obtained. It appears, therefore, that ivermectin is less effective in treating brugian filariasis than it is in treating bancroftian filariasis. This is surprising, since just the opposite result is seen with DEC treatment.

APPENDIX II.

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