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# THE ROLE OF POLYMERASE CHAIN REACTION TECHNIQUES FOR ASSESSING LYMPHATIC FILARIASIS TRANSMISSION

**Report of a workshop cosponsored by the World Health Organization and**
DBL-Centre for Health Research and Development, University of Copenhagen

Copenhagen, Denmark

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## ABBREVIATIONS

| | |
|---|---|
| ABR | annual biting rate |
| ADL | adenolymphangitis |
| AIBR | annual infective biting rate |
| ATP | annual transmission potential |
| CDC | Centers for Disease Control (USA) |
| CMFL | community microfilaria load |
| DANIDA | Danish International Development Agency |
| DBL | Danish Bilharziasis Laboratory |
| DEC | diethylcarbamazine citrate |
| EU | European Union |
| GPELF | Global Programme to Eliminate Lymphatic Filariasis |
| GPS | Global Positioning System |
| ICT | immunochromatographic test |
| IHRD | Institute for Health Research and Development |
| IU | implementation unit |
| L3 | third-stage larva |
| LF | lymphatic filariasis |
| LQA | lot quality assurance |
| MDA | mass drug administration |
| MF | microfilaria(e) |
| MoH | Ministry of Health |
| MX | molecular xenomonitoring |
| NIH | National Institute of Health (USA) |
| MLE | maximum likelihood estimator |
| NPELF | National Programme to Eliminate Lymphatic Filariasis |
| NTD | neglected tropical disease |
| OCP | Onchocerciasis Control Programme |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PELF | Programme to Eliminate Lymphatic Filariasis |
| PNG | Papua New Guinea |
| PMG | Programme Managers' Guidelines (WHO) |
| qPCR | quantitative PCR |
| RFV | recently fed vector |
| SEARO | South-East Asia Regional Office (WHO) |
| TAG | Technical Advisory Group |
| TDR | Special Programme for Research and Training in Tropical Diseases (UNICEF/UNDP/World Bank/WHO) |
| UMP | uniformly most powerful |

# 1. INTRODUCTION

## 1.1. Rationale for the meeting

An international workshop entitled "Lymphatic Filariasis: Use of PCR in Monitoring Transmission" was held on 7-10 November 2006. The purpose of the meeting was to discuss and standardize the use of PCR as a tool for monitoring the success or failure of mass drug administration (MDA) programmes and to aid in determining when MDA programmes can be terminated.

The specific objectives of the meeting were to:

- prepare a standardized protocol for infection monitoring by mosquito dissection, including a mosquito-sampling strategy;
- prepare a standardized protocol for infection monitoring by PCR analysis of mosquitoes, including a mosquito-sampling strategy;
- examine and list the criteria that should be used to determine when significant transmission has ceased in an endemic area;
- prepare a standardized sampling protocol to be used in determining that significant transmission has ceased in an endemic area.

The detection of lymphatic filarial parasites in mosquitoes by PCR and its potential use as a monitoring and evaluation tool in the Global Programme to Eliminate Lymphatic Filariasis (GPELF) has been discussed for several years[1,2,3,4,5]. During the TDR Scientific Working Group meeting on Lymphatic Filariasis (LF) in Geneva, May 2005, and at the subsequent meeting of the Technical Advisory Group (TAG-6) of the GPELF in Geneva, September 2005[6,7], it was emphasized that the use of PCR in molecular xenomonitoring (MX) should be evaluated as a possible tool to monitor progress of the GPELF. Members of the TAG indicated a need to streamline the available PCR technology to make it more useful and easier to implement on a global scale.

It was recommended that available expertise be brought together for a three-day meeting to review the PCR methodology and to make recommendations for quantifying field data in relation to mosquito catches and the presence of infective larvae (L3s) or other stages in a defined host-parasite-vector ecological system. It was felt that such an analytical approach to monitoring infections in the mosquito, along with available antigen and antibody tests, would give much needed, evidence-based scientific information towards verification of the absence of transmission in endemic areas where multiple rounds of MDA had been completed. This report summarizes the results of the meeting and the conclusions drawn on the use of PCR in monitoring filarial infection in mosquitoes. It also reports on standardized methods that can be used in the field.

## 1.2. DBL–IHRD as meeting hosts

Dr. Niels Ornbjerg, Director opened the meeting, presented the host institution and summarized the involvement of DBL in LF

#### *1.2.1. DBL – Institute for Health Research and Development*

- Small institution with total staff of 27
- Core funding from DANIDA, but with support from EU, NIH and Gates Foundation.
- Began 42 years ago as the Danish Bilharziasis Laboratory:
  - first studying snail vectors of schistosomiasis
  - next studying all aspects of schistosomiasis
  - then addressing a broader range of neglected tropical diseases (NTDs)
  - now has studies ranging from molecular taxonomy to health, social sciences and health systems research
- High visibility, especially in Africa and in capacity building
- Advances DANIDA's strategies and policies by:
  - aiming to contribute to sustainable health development
  - creating a research agenda that is 80% applied and 60% interdisciplinary and/or cross-sectoral

#### *1.2.2. DBL and LF*

- 20 years of seminal work in epidemiology, immunology, vector biology and community-based drug delivery
- Unparalleled capacity building for filariasis in Africa through courses, workshops and collaborative research
- This workshop is being convened because of the important need it fills and because of DBL's particular suitability for addressing this need and thereby helping WHO and the Global Alliance advance the goals of GPELF.

### 1.3. Principal conclusions of the workshop

#### *1.3.1. Mosquito dissection and sampling*

- Dissection of vector mosquitoes for third-stage larvae (L3) is at present the only proven method to estimate transmission to humans.
- Following infection/infectivity rates in vectors is a useful method to monitor transmission, but the practicalities of collecting sufficient numbers of mosquitoes may limit its value in determining transmission cessation.
- Transmission of lymphatic filariasis is a function of *both* the prevalence of mosquitoes with infective-stage larvae (infectivity rate) and the human-biting rate; therefore, transmission monitoring must also include estimates of human-biting rates.

- Mosquito surveys for monitoring infection/infectivity rates should be conducted at least six months after MDA.
- The transmission monitoring strategies recommended by GPELF (through antigen detection, usually by the immunochromatographic test card (ICT) and microfilaria detection) should continue to be followed while the role of vector-based surveillance approaches is evaluated.

### *1.3.2. Use of PCR on pools of mosquitoes for end-point assessment and post-MDA surveillance*

- MX and mosquito sampling assessment should focus on individual villages (or a cluster of villages when villages are small), rather than on implementation units.
- MX villages should be selected in areas where microfilaria (MF) rates have been reduced to low levels by MDA.
- Mosquitoes should be collected at least 6 months after the last round of MDA and when the target mosquitoes needed for testing are abundant.
- Timing of mosquito collections should be approximately the same as for MF testing so that MX data can be related to MF prevalence data.
- Testing should be on female mosquitoes (vector species only) that have recently fed (gravid, semi-gravid, or blood engorged).
- Mosquito collection methods need to ensure sufficient numbers for testing: gravid traps for *Culex* sp., light traps for *Anopheles*, BG traps for *Aedes*, and cattle shed collections for *Mansonia*.
- Traps should be distributed to cover all areas in study villages. A uniform number of pools/mosquitoes should be tested from each trap when possible. This will improve representation of all people in the village.
- The number of pools and mosquitoes for MX testing should follow power guidelines and sample size tables/graphs to target assessments of infection prevalence rates of 0.1, 0.25, 0.5, 1, and 2%.
- Provisional target thresholds for suspension of MDA are mosquito DNA rates by MX of < 0.25% for *Culex*, < 1% for *Anopheles*, and < 0.1% for *Aedes*. No provisional target rate for *Mansonia* has been suggested at this time.
- Parallel mosquito dissection studies should be performed when feasible to compare infection rates obtained by dissection and MX, and also so that these two parameters can be related to other factors such as MF and antigen prevalence rates in humans.

### *1.3.3. Standardized protocol for xenomonitoring by PCR analysis of mosquitoes*

- Definitive protocols for mosquito processing and PCR analysis were agreed upon (Appendix 7.4).
- An estimated MX processing rate per technician is 48 pools per day; at 20 mosquitoes per pool = 1000 mosquitoes per day = 5000 mosquitoes per week; 50 weeks = 250,000 mosquitoes per technician per year.

- Rate per instrument per year (given two technicians) = 500,000 mosquitoes per year
- Cost estimate: $4.00 per pool (may be less with discounts/donations from companies that produce the reagents). This is equivalent to $0.20 per mosquito.

#### *1.3.4. Next steps*

- The issues examined and resolved during this workshop can now be carried forward and translated into action through a Gates Foundation grant awarded to the Global Alliance coincident with this meeting and designed to address critical operational research issues now facing the GPELF
- Standardized xenomonitoring protocols can be put into research studies comparing them with other diagnostic approaches to monitor progress and provide surveillance for the GPELF

## 2. GLOBAL PROGRAMME TO ELIMINATE LYMPHATIC FILARIASIS

### 2.1. Current status of the GPELF

#### *2.1.1. People treated in 2005: 390 million*

- 90 million with 2-drug regimens
- 300 million with diethylcarbamazine citrate (DEC) alone (all in India, which in 2006 opted to use the more effective Alb-DEC 2-drug regimen for the future)
- SEARO and Mekong + countries in WPRO (Cambodia, the Lao People's Democratic Republic, Malaysia, Philippines, Viet Nam) are already reaching 60-70% of their target populations.

#### *2.1.2. Impact of MDA programmes*

- Generalization: there is significant impact of MDAs on reduction of microfilaraemia, with total clearance of MF in 60% of sentinel sites after only 2-3 MDAs.
- Exception: in certain situations five rounds of MDA have not been adequate to meet the criteria for stopping the MDAs.
- The number of MDAs needed appears to depend on the baseline infection level, drug coverage and the specific vector-parasite complex being targeted.
- MDA distribution system provides opportunity to deliver other large-scale interventions.

#### *2.1.3. Impact of LF disability prevention (via long-term, home-based self-care)*

- Significant reductions in frequency of adenolymphangitis (ADL) attacks and significant improvement in quality of life for lymphoedema patients have been documented.
- The self-care approach is amenable to being introduced through different operational models of community home-based care.

## 2.2. GPELF perspective on indicators for stopping MDA and post- intervention surveillance

### *2.2.1. Phases in the elimination time-line*

- The decision to stop MDA does not require that infection be *absent* from a community, only that it has reached a threshold where recrudescence does not occur.
- Surveillance post-intervention will be utilized to monitor for appearance of new infections.

### *2.2.2. Criteria for adopting a new or additional diagnostic tool*

- Tool attributes:
  - robust, standardized and replicable
  - available and affordable
- To replace an existing tool, it must be:
  - more sensitive/specific than the existing tool
  - more cost-effective
  - operationally more feasible
- As a supplement to existing tools it would have to increase significantly the confidence for decision-making in programmatic settings.

## 2.3. Current criteria for stopping MDA (WHO's guideline on Monitoring and Epidemiological Assessment of the PELF at IU level)

### *2.3.1. Prior to the 5th MDA*

- Check sentinel and spot-check sites (500 people per site as defined in the PMG) to ensure that prevalence of MF by blood smear is <1%.
- Add ICT testing of 2- to 4-year-old children to make sure that recent, new infections have not occurred.
- If all the above are true, add an additional 5-10 spot-check sites in areas expected to be at 'high risk'.

### *2.3.2. Post-5th MDA*

- Conduct "community Lot Quality Assurance (LQA) cluster survey" for ICT positivity in 300 children (2-4 years old) in high-risk areas: *no positives = <1% prevalence.*

- If the community LQA cluster survey is 'negative' (i.e., identifies no positives), conduct large-scale LQAs for ICT positivity in 3000 school-entry-age children sampled systematically from the total Implementation Unit (IU): *no positives = <0.1% prevalence.*
- If the large-scale LQAs of school-entry-age children are 'negative', then MDAs can be stopped.

## 2.4. Likely future changes in criteria for stopping MDAs

### *2.4.1. Modifications of target populations to be sampled*

- Children older than 2 to 4 years to be screened for ICT positivity pre-5$^{th}$ MDA
- Children older than school-entry age to be sampled in the large-scale LQAs post-5$^{th}$ MDA
- Use of vector populations?

### *2.4.2. Modifications in the sampling strategies and timings*

### *2.4.3. Changes in the indicators to be assessed*

- Use of antibody assays (especially for *B. malayi*)?
- Use of DNA assays (in human and/or vector populations)?

### *2.4.4. Modifications of target thresholds for decision-making*

- in human populations
- in vector populations

## 2.5. The role of PCR in detecting infection (and possibly assessing transmission) through xenomonitoring

### *2.5.1. In the past*

- No role for PCR-based diagnostics in the GPELF
- Largely experimental and operational research:
  - perfecting techniques
  - seminal pilot studies in Dominican Republic, Egypt, French Polynesia, Ghana, Haiti, India, Indonesia, Nigeria, Papua New Guinea, Tanzania and Trinidad

### *2.5.2. In the future*

- Course being charted at this meeting.
- Implementing the assessment of PCR-based xenomonitoring (charted at this meeting) is now possible through a new grant for operational research from the Bill and Melinda Gates Foundation to the Global Alliance to

Eliminate LF (GAELF) entitled, "Resolving the Critical Challenges Now Facing the Global Programme to Eliminate Lymphatic Filariasis":

- A key element of this grant is the direct comparison of nine available diagnostic assays (based on parasite, antigen, DNA or antibody detection in samples of blood, urine or mosquitoes) in situations where, after 5 or more MDAs, national programmes are considering stopping their MDA activities and initiating surveillance activities instead.
- Nine countries (selected to represent the principal different vector-parasite complexes of LF) will participate in this complex, but fully funded, multi-centre study employing identical, standardized, mutually agreed techniques.
- The approach to using PCR for xenomonitoring of the vector population in this multi-centre study will be based on the outcome of this present workshop, hence this workshop's very appreciable importance.

## 3. MOSQUITO DISSECTION AND SAMPLING FOR USE IN INFECTION MONITORING

### 3.1. Background

Transmission of LF has two components: transmission from human to the mosquito vector and transmission from the mosquito vector to the human host. Transmission can therefore be monitored and evaluated by measuring changes in infection status in either the vector or the human.

Direct detection of early stage larvae in the vector is indicative of both the presence of patent (circulating microfilariae) infections in humans and transmission of the parasites from human to vector. Detection of third stage larvae in mosquitoes is a measure of the potential for transmission from mosquito vectors to humans. Estimating transmission from mosquitoes to humans in the vector requires estimates of both the proportion of the vector species that is infected with third stage larvae and the amount of contact between the human and vector populations.

The most unequivocal direct measure of the infection status in vectors is the identification of larvae in known vectors by dissection. However, the accuracy and precision with which LF infections in vectors can be estimated may be limited by the ability to process sufficient numbers of mosquitoes by dissection, particularly when vector infection rates are low. Hence, there may be epidemiological situations when *indirect* measures of vector infection status are required.

The sensitivity and specificity of PCR for detecting DNA enables processing of pools of vectors, so that screening for the presence of filarial DNA in larger numbers of vectors is facilitated. The differential sensitivity of dissections for filarial worms and PCR detection of filarial DNA has led to differences in the estimates of infections in vector populations in endemic areas by up to a log order. In addition, recent laboratory experiments have identified filarial DNA but no parasites in non-vector

mosquitoes for up to three weeks after exposure to microfilariae. The implications of these laboratory experiments for the interpretation of PCR analyses of mosquitoes collected during monitoring activities for LF elimination programmes are still uncertain.

### 3.2. Strategies for mosquito collection in different parasite/vector epidemiological settings for monitoring transmission (including the sampling strategy)

Estimating the infection status in vectors requires vector sampling strategies (vector surveillance/monitoring) that include both a method of collecting adequate numbers of mosquitoes and ensuring that the mosquito samples are representative of the entire vector population. In selecting a surveillance strategy, it is important that the bias of the sampling method remain constant during the time period that the population is monitored. Hence, programmes should be cognisant of factors, including vector control interventions that might be implemented, which would affect sampling bias. For instance, indoor residual spraying and distribution of insecticide-treated mosquito nets will change both the numbers of mosquitoes collected in indoor resting collections and the proportion of these mosquitoes that will have fed on humans. By definition, vector surveillance/monitoring implies longitudinal collections to determine if there are changes in infection status. In the absence of background data, year-long vector collections should be undertaken to determine seasonal patterns of transmission in order to optimize sampling.

While a large number of mosquito species are recognized as vectors of lymphatic filarial parasites, there are only a few tools available for sampling mosquitoes. The use of standard collection methods in different geographical areas with similar vectors should facilitate comparison of these tools and their roles in helping to interpret the effectiveness of LF elimination programmes. Table 1 records experiences with the numbers of vectors that have been collected in different geographical locations using different sampling methods.

Techniques that capture gravid or blood-fed mosquitoes are most useful. Landing catches should be conducted using known LF negative individuals. Implementation of vector surveillance will need to consider the sampling bias of the collection method (see above) and the capacity of the sampling method to obtain an adequate number of vectors (which may be limited both by the biology of the vector and by financial and operational considerations). Landing catches are labour-intensive, require supervision and may require ethical approval. Gravid, BioGents and light traps have costs associated with their purchase, operation and maintenance.

Table 1. Tools for collecting vectors of lymphatic filariasis by genera

| Vector | Sampling tool | Mosquitoes/collection period Estimated average range | Country |
|---|---|---|---|
| *Culex* | Indoor resting | 0-50/house/day | Egypt |

| | | | |
|---|---|---|---|
| | catches | 0-10/house/day (rural)<br>10-25/house/day (urban) | India<br>India |
| | Gravid traps | 0-958/trap/day (mean=80) | Egypt |
| | Light trap | 0-21/trap/night (mean=4)<br>25-90/trap/night | Kenya/Tanzania<br>Zanzibar |
| *Anopheles* | Landing catches | 10-50/person/night<br>30-200/person/night | Ghana<br>PNG |
| | Light traps | 0-50/night<br>5-10/trap/night<br>10-20/trap/night<br>5-75/trap night (mean=38) | PNG<br>Ghana<br>Vanuatu<br>Kenya/Tanzania |
| | Indoor resting | 0-20/house/day | PNG |
| *Mansonia* | Indoor resting | 5-10/house-cattleshed/day (rural) | India |
| *Aedes polynesiensis* | Landing Catches | 10-100/30 min collection<br>2-40/10 min collection | Am. Samoa<br>Fr. Polynesia |
| | BioGents Trap | 5-40/trap day | Am. Samoa |
| *Aedes samoanus* | Landing catches | 5-10/person/night | Samoa |

### 3.3. Options for mosquito dissection

Dissection options include:

1. dissection of fresh, live, unstained mosquitoes and immediate examination;
2. dissection and examination of thawed cold-killed mosquitoes that have been stored frozen;
3. dissection of live unstained mosquitoes after allowing them to dry prior to staining;
4. preservation of fresh mosquitoes in alcohol prior to staining, followed by dissection and examination;
5. dissection of fresh engorged mosquitoes followed by red blood cell lysis prior to staining;
6. mass dissection.

- The first option, dissections of live mosquitoes followed by immediate examination for filarial larvae, is limited by the number of mosquitoes that can be processed in a day (i.e., by the number and efficiency of technicians available).
- The second option is the same as the first, but allows storage of many mosquitoes during substantial periods.
- The third option requires care to be taken in using clean slides to minimize the risk of losing material from slides.
- The third option has the advantage of allowing field workers to concentrate on the collection of mosquitoes by minimizing the handling of specimens in the

field. Laboratory technicians can concentrate on staining, dissecting and examining mosquitoes.

- Examination of stained mosquitoes may enhance the ability to detect early-stage larvae by microscopic examination. However, since blood-engorged mosquitoes are not amenable to immediate haematoxylin staining and dissection, evaluation of mosquitoes collected in indoor resting collections is restricted to examination of heads and thoraxes. If examinations of recently fed mosquitoes for the presence of microfilariae are required, abdomens can be dissected in saline, and examined immediately. It is recommended that heads, thoraxes and abdomens be separated prior to dissection by any of the above methods.
- Finally there are mass dissection techniques for third stage larvae in which mosquitoes are crushed in bulk, submerged in saline (infective larvae wriggle out), and then larvae are collected at the base of a funnel. Mass dissection can then process a large number of mosquitoes. However, the sensitivity of this technique may not be appropriate when infection rates are low.

There is a general consensus that dissection of live unstained mosquitoes for the detection of third stage larvae is sensitive and easy to perform. Similarly for detection of microfilariae in engorged mosquitoes, dissection of unstained live mosquitoes is appropriate, being both sensitive and easy to perform. Agreement was not reached on the merits of staining to detect all stages in all areas: for experienced groups, larval infection rates *can* be reliably detected in unstained mosquitoes. Observations of infections in *Culex quinquefasciatus* in India suggest that third stage larvae are not found when the all-stage infection rate falls to 1% or less. This is an observation that needs to be confirmed elsewhere.

### 3.4. Rate of processing mosquitoes by dissection

The consensus is that a skilled technician should be capable of processing 50 to 100 mosquitoes per day by dissecting individual mosquitoes for detection of all stages of the parasites. This translates to approximately 12,000-24,000 mosquitoes per year per technician. Significantly greater numbers of mosquitoes can be processed for third-stage larvae alone by mass dissection.

### 3.5. Epidemiological settings for collection of adequate numbers of mosquitoes

At least four factors will determine if a sufficient number of mosquitoes can be collected to monitor transmission.

- The expected level of infection in the mosquito population will affect the size of the sample that must be analysed. Indeed, the number of mosquitoes required to determine whether transmission has ceased at the completion of MDA might need to be determined by modelling. This end-point is likely to be different for different vector systems and will require significantly more mosquitoes to be examined than is necessary for determining if transmission is diminishing during the early phases of an MDA programme.

- The second factor that will determine if adequate numbers of mosquitoes can be collected is the resources available for vector surveillance (e.g., for traps, field workers and laboratory technicians). In many countries, the capacity of MoHs to undertake vector surveillance activities has greatly diminished during recent years.
- A third factor is the size of the vector population. This is a function of the innate capacity of the ecology to support mosquito populations as well as the level of vector control that may be operating on the population. For example, in Tanzania, the use of polystyrene beads in pit latrines significantly reduced the vector population, thereby rendering problematic the collection of large numbers of mosquitoes in order to detect low infection rates.
- The fourth component is the duration of the optimal season for mosquito availability and its relationship to the timing of MDA.

### 3.6. Conclusions and recommendations on sampling/dissecting mosquitoes for infection monitoring

- Dissection of vector mosquitoes for third-stage larvae is presently the only proven method to estimate transmission to humans.
- Following infection/infectivity rates in vectors is a useful method to monitor transmission. The practicalities of collecting a sufficient number of mosquitoes may limit its usefulness for determining transmission cessation, particularly when sampling methods collect mosquitoes outside of houses.
- Transmission of lymphatic filariasis is a function of both the prevalence of mosquitoes with infective stage larvae and the human-biting rate. Monitoring transmission must include an estimate of human-biting rates.
- The relationship between total larvae infection rates and third stage larvae infection rates (infectivity rates) in the major vector systems should be investigated. Confirmation of the Indian observation that transmission ceases when the all-stage larval infection rate falls to 1% should be undertaken in further studies in other countries.
- Mosquito surveys for monitoring infection/infectivity rates should be conducted at least six months after MDA.
- Collection of indoor resting mosquitoes is the most appropriate technique for evaluation of xenomonitoring in many areas. However, use of insecticide-treated mosquito nets and indoor residual spraying will increasingly limit the areas where this collection technique can be used.
- The activities of other vector control programmes will impact LF vector indicators including LF infection rates and vector densities. The impact of these activities on LF transmission and LF vector surveillance needs to be considered and monitored.
- Transmission monitoring strategies recommended by the GPELF (through ICT and microfilariae detection) should continue while the role of vector surveillance-based approaches is evaluated.

## 4. USE OF PCR ON POOLS OF MOSQUITOES FOR END-POINT ASSESSMENT AND POST-MDA SURVEILLANCE

### 4.1. Current understanding of PCR detection of filarial DNA in mosquitoes

- Molecular xenomonitoring (MX) detects filarial DNA in mosquitoes.
- A positive mosquito MX test indicates the presence of patent filarial infections in the human population and is much more sensitive for this purpose than dissection. Although it provides a measure of population infection at a given point in time, no national LF elimination programme is currently using either MX or dissection to assess the efficacy of MDA or as an end-point. However, *dissection* has been used in pilot studies for this purpose in Tahiti, Ghana, India and Papua New Guinea; and *MX* has been used in small projects to monitor effects of MDA in Brazil, Egypt, Tanzania, Nigeria, Haiti, the Dominican Republic, and Papua New Guinea. MX has also been used to verify lack of active LF in Trinidad and Tobago.)
- Mosquito *dissection* can distinguish filarial infection and infectivity, but it is less useful in low prevalence areas or after MDA when mosquito infection and infectivity rates are very low. MX (i.e., PCR) is not specific for L3, and it does not indicate infectivity. Currently, there is no PCR method available to detect L3 infectivity.
- MX with mosquitoes can be used to detect the presence of filarial parasite infections in humans before or after MDA (which is especially valuable since the procedure is noninvasive and does not require blood collection or active cooperation from endemic populations), *but it does not directly reflect transmission.*
- PCR generates higher values for parasite rates in mosquitoes than dissection. Studies from PNG suggest that this may be due to MF or MF remnants that are not detected by dissection.
- PCR can detect filarial DNA in mosquitoes that do not contain live or dead parasites visible by microscopy. While it is possible that mosquitoes may take up "free parasite DNA" from human blood that is not associated with intact MF, additional research is needed to test this hypothesis.
- Filarial DNA can remain detectable in *non-vector* mosquito species for weeks after mosquitoes feed on MF-positive blood. (Parasites are rarely detected by *dissection* in non-vector mosquitoes later than one day after feeding.)
- Real-time PCR is more sensitive than conventional PCR (which detects PCR products by agarose gel electrophoresis), but its main advantages are lower contamination risks and higher throughput capacity compared to conventional PCR. Real-time detection of PCR products saves labour and avoids the use of ethidium bromide. However, laboratory infrastructure requirements (including expensive equipment, ready access to kits and reagents, and the need for highly trained personnel) favour the operational model of establishing a network of regional reference laboratories for molecular xenomonitoring/real-time PCR work.
- Transmission is very unlikely if no filaria-positive mosquitoes are detected by MX, but this assumes that adequate samples have been collected and tested. An

important challenge is to establish limits or targets for mosquito DNA rates that indicate a negligible risk of transmission and which can be used as an end-point for MDA.

- Pool Screen (developed by Dr C. Katholi to 'translate' PCR positivity into mosquito infection rates) has a new version for hypothesis testing to assess targets/end-points. It requires power calculations to assure use of sufficient sample sizes (i.e., the number of mosquitoes and pools needed to show that filarial DNA rates in mosquitoes are less than 2%, 1%, 0.5%, 0.25%, 0.1%, etc. (pools should consist of 10-25 mosquitoes each).
- Data from Egypt suggest a provisional end-point DNA rate of $\leq 0.25\%$ (upper confidence limit) by PCR for *Culex* vectors. This correlates with a MF prevalence rate of $\leq 1\%$ by membrane filtration of 1 ml venous blood. Additional studies are in progress to verify/refine this end-point.
- While PCR monitoring should be most useful for end-point assessment and for post-MDA surveillance, it could also be a useful alternative for mapping *Brugia* endemicity.

### 4.2. Recommendations regarding PCR detection of filarial DNA in mosquitoes

- All available MX data linked with other measures of infection in mosquitoes and humans should be brought together in order to define the range of rates of DNA in mosquitoes occurring in areas with ongoing LF transmission.
- Existing data from pilot studies should be coupled with expert opinion to develop provisional MX protocols for end-point and surveillance purposes for different vectors
- Procedures should be established for use of MX as a post-MDA surveillance tool for early detection of LF resurgence. Available data are not sufficient to make firm recommendations, but pilot studies are promising enough to warrant further investigation.
- MX and mosquito sampling should focus on individual villages (or a cluster of villages when villages are small) rather than on larger implementation units.
- MX villages should be in areas where MF rates have been reduced to low levels by MDA.
- Mosquitoes should be collected at least 6 months after the last round of MDA and when the target mosquitoes needed for testing are abundant.
- Traps should be distributed to cover all areas in study villages. A uniform number of pools/mosquitoes should be tested from each trap when possible in order to improve representation of all humans in the village.
- Parallel mosquito dissection studies should be performed when feasible to compare infection rates obtained by dissection and by MX and also so that these two parameters can be related to other parameters such as MF and antigen prevalence rates in humans (at least one site should be studied for each major vector/parasite complex).
- The timing of mosquito collections should be approximately the same as that for MF testing so that MX data can be related to human MF prevalence.

- Testing should be on female mosquitoes (vector species only) that have recently fed (gravid, semi-gravid or engorged). Studies should focus on a single dominant vector species when this is feasible.
- Mosquito collection recommendations are to be found in Appendices 7.1 and 7.4.
- The number of pools and mosquitoes for MX testing should follow power guidelines and sample size tables/graphs for target infection prevalence rates of 0.1, 0.25, 0.5, 1, and 2% (Appendix 7.2.1).
- Provisional mosquito positivity thresholds by MX for stopping MDA are < 0.25% for *Culex*, < 1% for *Anopheles*, and < 0.1% for *Aedes* (no provisional target rate for *Mansonia* at this time). MX assessments should be powered to use these values as upper confidence limits for infection prevalence estimates.
- Protocols should also be developed to determine whether LF is absent from a country

## 5. STANDARDIZED PROTOCOL FOR XENOMONITORING BY PCR ANALYSIS OF MOSQUITOES

### 5.1. Protocol development process

The essential elements of xenomonitoring working protocols for using PCR to detect parasite DNA were defined at the meeting. Later (over the course of 6 months) detailed protocols were drafted and agreed by meeting participants (see Section 7.4). The essential elements of the mosquito collection and sampling protocols can be found in Sections 3 and 4 (above), with fuller SOPs in Section 7.4. Essential elements of the laboratory processing of specimens are listed below (Section 5.2), while fuller SOPs can be found in Section 7.4.

### 5.2. Laboratory MX protocol essential elements

#### *5.2.1. At the point where the sample catch comes into the 'field laboratory'*

- Kill the mosquitoes (by putting mosquitoes in containers in the sun for 5-10 min or by freezing).
- Sort by trap: mosquitoes kept for testing will be female, vector and blood-fed/gravid/half gravid (where possible, collecting only the major vector species for these studies).
- Dry the mosquitoes; there are multiple ways of doing this (oven, sun, etc.).
- Label with country code, number and date corresponding to a data sheet with collector's name and trap number, location, date and number of mosquitoes.
- Mail to reference laboratory for processing.

#### *5.2.2. At the point where the samples come into the reference laboratory*

- Sort the tubes by trap over the total time period of collection.

- Combine all mosquitoes from a single trap.
- Randomly select groups of 20 mosquitoes (the designated pool size) into 2 ml round bottom tubes (number of pools per trap and total number of mosquitoes assessed depends on the study).
- Use BB grinding method to break up the mosquitoes and parasites.
- DNA extraction method: Optimized Qiagen Method using the Qiagen DNeasy Kit).
- Real-time PCR assay and detection.
- Record data results and send back to programme managers to help in the programme evaluation.

### 5.3. Laboratory processing capacity and costs

- Rate per technician per year:
  - Estimated processing rate per technician is 48 pools per day @ 20 mosquitoes per pool = 1000 mosquitoes per day = 5000 mosquitoes per week; 50 weeks = 250,000 mosquitoes per technician per year.
- Rate per instrument per year:
  - With two technicians = 500,000 mosquitoes per year
- Cost estimate per pool: $4.00
- Cost estimate per mosquito: $0.20
  - Costs could be reduced with discounts/donations from companies that produce the reagents.
- Note: these cost estimates do not include labour, since labour costs depend on where the work is done.

## 6. CONCLUSIONS

The workshop "Lymphatic Filariasis: Use of PCR in Monitoring Transmission" was highly successful in initiating development (now complete) of protocols for mosquito collection, dissection and PCR detection of vector-borne filarial parasites. The two original objectives that were more complex – i.e., 1) defining the *criteria* to be used to determine when significant transmission has ceased in an LF endemic area, and 2) developing standardized sampling protocols for use in determining when those criteria have been reached – require further deliberation, but at least the appropriate tools necessary for addressing these objectives are now available and well defined.

The timing of the workshop was particularly opportune, as shortly thereafter the Global Alliance received a grant from the Bill and Melinda Gates Foundation to focus on "Resolving the Critical Challenges Now Facing the Global Programme to Eliminate Lymphatic Filariasis." An important objective of this grant is the comparison of nine available diagnostic assays (based on detecting parasites directly or on detecting parasite antigen, parasite DNA or anti-parasite antibodies). Human blood, human urine, and mosquitoes will be collected in nine different countries where, after five or more MDAs, national programmes are considering stopping their MDA activities and initiating surveillance activities instead.

As a result of the planning and assessment initiated by this workshop, PCR-based molecular xenomonitoring can be effectively utilized in these comparative studies, and its practical potential for use as a critical tool for the GELPF can be determined. At the conclusion of this assessment, the relative value of using PCR on mosquitoes, compared to other measures for helping to evaluate the effectiveness of MDA programmes, should finally be clear.