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MICROSCOPIC VISUALIZATION OF *WUCHERERIA* AND *BRUGIA* LARVAL STAGES IN INTACT CLEARED MOSQUITOES

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Abstract. Over the past several decades, epidemiologic data from filarial vectors typically has been obtained by mass dissection or by dissection of individual specimens. The former is quick and easy to do on large numbers of insects but provides no information on the frequency distribution of infection, presence of early developmental stages, or larval location; the latter is labor-intensive and tedious. We describe a new technique that can provide data comparable to those obtained by individual dissection, including calculation of infection and infective rates, and this technique is easy enough to accommodate large numbers of insects. Brief treatment of ethanol-fixed, intact mosquitoes in sodium hypochlorite, followed by treatments in increasing concentrations of ethanol and an organic solvent allowed microscopic visualization of filarial larvae within the abdomen, thorax, head, and proboscis of *Brugia malayi*–infected *Aedes aegypti* and *Wuchereria bancrofti*–infected *Anopheles punctulatus*. We compared the classic techniques to our technique using *Ae. aegypti* infected by feeding on jirds with *B. malayi* microfilaremias. Comparisons of the infective rate, total number of infective stage larvae (L3s) observed, and locations of L3s showed that this new technique was comparable to the established methods, while being faster and more precise in determining the location of larvae.

Information collected from insect vectors is an integral part of filariasis surveillance and control efforts. These data are used to monitor infection dynamics in vectors and to determine the transmission potential of human filariasis. It is particularly useful for assessing the impact of filariasis control measures such as community-wide chemotherapy. Using the insect filarial burden and larval location, infection rates (the percentage of insects harboring developing filariae)[1] and infective rates (the percentage of insects containing infective stage larvae [L3s])[1] as well as monthly and annual transmission potentials can be calculated. Transmission potentials are perhaps the most useful of these data because vector biting rates are included in the calculation.[2] For several decades, dissection of individual insects has been the standard entomologic method for acquiring these data. The technique has remained useful because of its simplicity and the precise information that it provides concerning presence, location, and developmental stage of larvae. Unfortunately, this approach is labor-intensive and limited by the time that it takes to dissect each insect. These problems tend to reduce the number of insects that can be evaluated in a study or experiment and may render the method unsuitable for monitoring transmission dynamics when infection rates are very low or when long-term, continual monitoring is needed.

An alternative technique that has been used is the mass dissection or crushing of mosquitoes.[3,4] This technique has the capacity to handle large numbers of insects, more than may be possible by individual dissection, but it only detects motile larvae, mostly L3s. All information concerning the actual numbers and locations of larvae in each mosquito is lost. Therefore, mass dissection is not suitable for determining infection and infective rates or monitoring the transmission dynamics over time. However, it has been suggested that information on the average number of L3s recovered per mosquito, as obtained by mass dissection, is sufficient to estimate the transmission risks from vectors.[4]

During work with live *Chrysops atlanticus* infected with *Loa loa*, it was noted that filarial larvae developing in the fat bodies and L3s within the hemocoel could be directly visualized with a compound microscope (Yates JA, unpublished data). These observations and the recent description of a staining technique to visualize nematode parasites within agricultural pest insects[5] prompted us to try visualizing *Brugia malayi* in infected *Aedes aegypti*. Because of the dark pigmentation of the *Ae. aegypti* exoskeleton, the described staining technique was unsat-

isfactory. However, the use of sodium hypochlorite to bleach the cuticle allowed visualization of filariae and eliminated the need for any stain. The microanatomic structures of the nematode provided sufficient contrast to easily identify the filarial larvae. Herein we describe a technique that combines simplicity with the capacity to process large numbers of insects without losing important data. This method may be used independently or as an initial screening to determine which insects require dissection or further study. In either circumstance, large enough numbers of insects could be processed to produce statistically significant results even in areas with low infection rates.

A fundamental problem in evaluating filarial infections in vectors has been the presence of filariae of wild and domestic animals in these insects. Dissection of vectors and recovery of larvae for species identification by morphology[6] may be an unavoidable necessity until alternate methods can be found for species identification. Our technique could allow the processing of large numbers of insects and limit the dissection to those vectors identified as infected.

To evaluate and validate our technique, we compared it with individual dissection and mass dissection using *B. malayi*–infected *Ae. aegypti.* We also visualized *Wuchereria bancrofti* larvae in *Anopheles punctulatus* collected in Papua New Guinea.

MATERIALS AND METHODS

Aedes aegypti (Liverpool, blackeye-strain) mosquitoes were raised in the laboratory and females were allowed to feed to repletion on inbred jirds, *Meriones unguiculatus,* with moderate *B. malayi* microfilaremias.[7] Blood-fed females were held for 14 days before being randomly separated into three groups of 125 for evaluation by individual dissection, mass dissection, or clearing.

For mass dissection, mosquitoes were stunned, placed on gauze on a glass plate, and crushed by rolling with a glass tube. The gauze containing the mosquitoes was then transferred to a wire screen in a funnel containing medium. The motile L3s were allowed to swim away from the debris and settle to the bottom, where they were collected after approximately 90 min.[8]

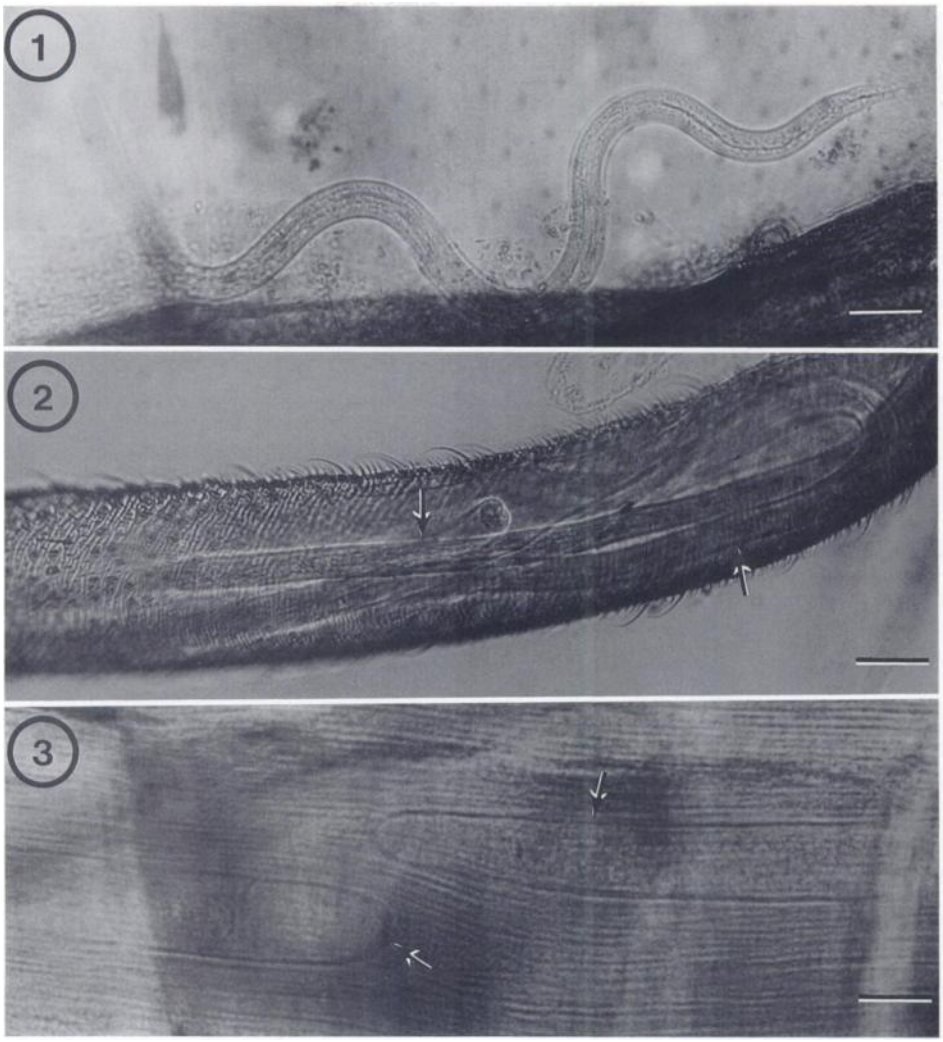
Mosquitoes for individual dissection or clearing were aspirated into 70% ethanol for fixation before being processed further. Mosquitoes to be dissected were either stained with Meyer's hematoxylin or placed directly in concavity slides with saline and dissected in the classic manner.[9] The head, thorax, and abdomen were teased apart and each body part was dissected individually. Dissected tissues were then viewed using 30× and 100× magnification to count and evaluate larval stages.

Mosquitoes were cleared and mounted using the following process. Ethanol-fixed mosquitoes were placed in a stainless steel mesh tea-ball (Scandicrafts. Inc., Camarillo, CA) and immersed in 5.25% NaOCl for various times depending upon species, *Ae. aegypti* for 45 min and *An. punctulatus* for 25 min. They were then washed twice with 70% ethanol (3 min/wash), and dehydrated for 30 min each in 80%, 95%, and absolute ethanol. The mosquitoes were then immersed in an organic solvent for 10 min to remove lipid deposits. Three organic solvents were used to clear lipid deposits. Methyl salicylate, toluene, or Polyclear (Polysciences, Inc., Warrington, PA) worked equally well. From the standpoint of laboratory safety, Polyclear was preferred due to its low toxicity and low volatility. The insects were then mounted on slides using an organic-solvent-based mounting media, either Permount (Fischer Scientific Co., Fair Lawn, NJ) or Cytoseal (Curtin Matheson Scientific, Inc., Wood Dale, IL). Cover slips were applied and gently pressed to contact but not crush the insects. Slides were viewed with a compound microscope using 100× and 400× magnification and examined for larval stages. Alternatively, mosquitoes can be stained with hematoxylin[9] before clearing in NaOCl and then viewed in temporary glycerine mounts, which facilitates subsequent removal of specimens.

A small batch of 50 wild-caught *An. punctulatus* was the kind gift of Dr. James Kazura (Department of Geographic Medicine, Case Western Reserve School of Medicine, Cleveland, OH). They were collected in a *W. bancrofti*–endemic area of the East Sepik Province, Papua New Guinea in January 1993. These mosquitoes were shipped to the United States in 70% ethanol containing 5% glycerol (v/v) and subsequently cleared, dehydrated, and examined by the process detailed above.

RESULTS

Representative photomicrographs showing *B. malayi* and *W. bancrofti* larval stages in intact,



FIGURES 1–3. Photomicrographs of cleared intact mosquitoes showing filarial larvae **(arrows)** in situ. **1,** *Brugia malayi* infective stage larvae (L3s) within the abdominal hemocoel of *Aedes aegypti* (bar = 45 μm). **2,** *Brugia malayi* L3s packed into the proboscis of *Ae. aegypti* (bar = 42 μm). **3,** *Wuchereria bancrofti* second stage larvae within thoracic flight muscles of wild-caught *Anopheles punctulatus* (bar = 20 μm).

cleared mosquitoes are presented in Figures 1–3. Locating the larvae within cleared mosquitoes was surprisingly simple. Infective stage larvae were seen within the hemocoel of the abdomen, thorax, legs, head, and proboscis of cleared mosquitoes. Recognizing L3s was straightforward because of their characteristic shape and extracellular location and could often be done with only 40× magnification. Larvae within the abdomen were easily distinguishable from the digestive and reproductive structures (Figure 1). Within the thoracic hemocoel, L3s were readily distinguishable from the respiratory tracheal system, which is frequently branched and comprised of supporting ring-like structures. The L3s in the neck and head areas were clearly distinguishable from the digestive tract, nerves, and musculature, and in the proboscis, L3s were seen in tightly packed bundles (Figure 2). First and second stage larvae (L1s and L2s) were seen within both the longitudinal and transverse flight muscles of the thorax (Figure 3). Upon clearing, flight muscles appeared as uninterrupted bands within their respective focal planes and this al-

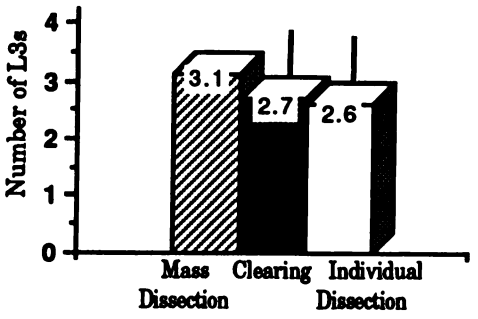


FIGURE 4. Means and standard deviations of infective stage larvae (L3s) found in laboratory-infected *Aedes aegypti* determined by three methods ($P > 0.95$, by analysis of variance).

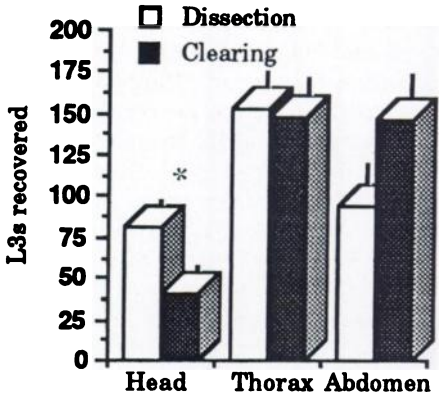


FIGURE 5. Distribution (mean and SD) of infective stage larvae (L3s) by location in laboratory-infected *Aedes aegypti* determined by two methods (*$P < 0.01$, by analysis of variance).

lowed easy identification of the intracellular larvae. First stage larvae were distinct, being short, fat, and sausage-like, while the L2s were longer and narrower.

To compare the number of L3s identified by each method, 125 bloodfed *Ae. aegypti* were processed using each technique. There was no significant difference in the mean numbers of L3s found per mosquito using the three methods (Figure 4). Note that the data from each method was so similar that there was only a 0.1–0.5 difference in L3s among the techniques. Information on the location and frequency distribution of L3s was obtained by the dissection and clearing methods. The locations of L3s found by dissection and by clearing were significantly different ($P < 0.01$, by analysis of variance, Figure 5). Dissection indicated that more L3s were in the head and fewer were in the abdomen than were observed by direct visualization of the cleared mosquitoes. However, the frequency distribution of L3 burdens determined by either method was similar (Figure 6). Using data obtained by dissections, the calculated infection rate was 71.2% and the infective rate was 70.4%. By the clearing method, the infection rate was 68.9% and the infective rate was 66.4%

The 50 wild-caught *An. punctulatus* from Papua New Guinea were processed by the clearing method and analyzed for the presence of larvae. From this small group, we calculated an infection rate of 12% and an infective rate of 6%. All larvae visualized were morphologically indistinguishable from *W. bancrofti*.[6]

DISCUSSION

The classic methods for evaluating filarial transmission, individual dissection and more recently mass dissection, have been effective for obtaining close approximations of transmission levels.[10–14] However, both of these methods have characteristics that constrain their use in repeated or continual close monitoring of infection dynamics in vectors. Individual insect dissection provides more useful data than mass dissection. Unfortunately, it is labor-intensive, tedious, and may damage or fragment filarial larvae.[3, 15–17] Mass dissection has enhanced the collection of highly motile larvae and is useful when the in-

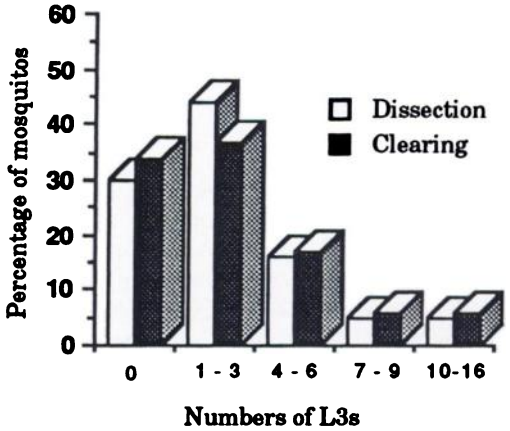


FIGURE 6. Frequency distribution of infective stage larvae (L3s) in laboratory-infected *Aedes aegypti* determined by two methods.

fective rates are extremely low, for example, one L3 per several hundred insects.[11] However, this technique is not useful for identifying the number of infected insects nor for detecting the relatively inactive L1 and L2 stages. *Onchocerca volvulus* L3s are not reliably detected in this way, probably due to their low motility.[18] Thus, rates of infection based on this technique may be flawed. None of these methods detect microfilariae within the digestive tract. These disadvantages are compounded by the fact that naturally occurring infection rates in potential vectors are low, even in areas in which the incidence of human infection is high.[19] Low baseline infection rates in vector populations require that large numbers of potential vectors be studied at each sampling if significant fluctuations in transmission potential are to be recognized. Repeated evaluation of such large numbers of insects may preclude the use of the individual dissection method.

Detection of statistically significant changes in infection and infective rates in vectors during and after intervention attempts is desirable. Changes in infection rates in vectors may be recognized years before changes in the incidence of human infection might be documented. Long-term surveillance of transmission potentials may be necessary to determine the duration of chemotherapy effects and the need for additional intervention. Antimicrofilarial agents such as diethylcarbamazine and ivermectin, which are currently being used in community-wide filariasis control campaigns, appear to lower infection rates in vectors.[12–14] Extremely low microfilaremias have been shown to perpetuate transmission[20] and in certain cases, the success rate of individual ingested microfilariae may increase as the number ingested decreases.[21]

Using a new clearing technique, we have been able to observe microfilariae, L1s, L2s, and L3s in experimentally infected laboratory-reared *Ae. aegypti* and also in naturally infected wild-caught *An. punctulatus*. This method is simple and insects can be processed in large groups in a relatively short time. The mean number of larvae found by each of the three methods varied by less than 0.5 L3 per mosquito. The clearing technique also produced nearly identical data to that obtained by individual dissection when frequency distribution was compared, although distributions of L3s by location differed.

We concluded that the clearing method could yield infection data similar to that obtained by individual dissection without the tedium and excessive labor. A further advantage of this new method was its ability to pinpoint the exact location of L3s. In the intact mosquito, larval positions were easily determined, especially those at the junctions of major body parts. An important difference that we found from individually dissected versus cleared mosquitoes was the numbers of L3s in the head. This discrepancy appeared to be an error resulting from dissection of the mosquito. During blunt dissection of the insect, larvae located in the thoracic hemocoel near the neck were caught in tissues that were removed along with the head. This consistently inflated the number of L3s identified as being in the head. A similar occurrence was noted for larvae at the thorax-abdomen junction. If L3s in the head are used as the basis for calculating monthly and annual transmission potentials, data from individual dissection may skew the statistic. Using intact mosquitoes to determine the larval locations may reveal that transmission potentials calculated with data from heads only were inflated.

Although we have studied the use of clearing in *Anopheles* and *Aedes* mosquitoes, its use may be extended to other types of insect vectors. In our laboratory, we were able to clear and see into a small number of *Simulium ochraceum* that were kindly provided by Dr. Mark L. Eberhard (Centers for Disease Control and Prevention, Atlanta, GA). These black flies were wild-caught in an *Onchocerca*-endemic area of Guatemala but they were not infected. We were able to clearly visualize individual flight muscle bands, retained or developing eggs, residual blood, and internal structures, which suggests that the clearing technique may be useful for monitoring *Onchocerca* infections in black flies.

This clearing method may be a practical way to collect entomologic data in filariasis research, preserving the simplicity of the classic methods while increasing the capacity to observe large numbers of insects. The usefulness of this technique in the field remains to be determined.

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