POOL SCREEN POLYMERASE CHAIN REACTION FOR ESTIMATING THE PREVALENCE OF *ONCHOCERCA VOLVULUS* INFECTION IN *SIMULIUM DAMNOSUM SENSU LATO:* RESULTS OF A FIELD TRIAL IN AN AREA SUBJECT TO SUCCESSFUL VECTOR CONTROL

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Abstract. Detection of infective parasites in the vector population can be an early indicator of recrudescence in areas freed of new cases of onchocerciasis. However, dissection of vector black flies is inefficient in areas subject to effective control. Recently, a polymerase chain reaction (PCR)–based assay has been used to detect a single *Onchocerca volvulus*–infected black fly in pools containing large numbers of uninfected flies. This method had not been validated on wild-caught black flies in an area subject to effective vector control. Here, we report a method of restricting the pool screen PCR assay to infectious parasites and the results of a field test in an area subject to long-term vector control. The prevalence of infection determined by dissection did not differ from that determined by pool screen PCR. The results suggest that the PCR assay may be a useful tool for epidemiologic surveillance for *O. volvulus* infection.

One of the major challenges of the Onchocerciasis Control Program (OCP) in West Africa is to place the 11 participating countries in a position to be able to take over its residual activities when the program comes to an end in 2002. At that time, epidemiologic surveillance and ivermectin treatment will remain the residual activities to be implemented by the countries. To ensure the success of these activities, the program must make available to the countries tools and methods for detecting recrudescence of *Onchocerca volvulus* infection that are both efficient and acceptable to the endemic population.

The most direct method of surveillance for *O. volvulus* recrudescence is to detect new infections in the endemic population. To this end, several methods are being tested to replace the skin snip method, which is becoming less and less acceptable to the endemic population.[1] In addition to conducting surveillance activities to detect new infections, it is also useful to detect *O. volvulus* infective larvae in the vector population. This is because the presence of *O. volvulus* infective larvae in the vector population is a direct demonstration that transmission is occurring, providing early warning that new infections may be developing. This is important because the prepatent period for the infection in humans is 18 months to two years.[2] Measurements of transmission can thus be used to rapidly identify potential areas of recrudescence of infection.

Onchocerca volvulus transmission has historically been measured through dissection of wild-caught vector black flies.[3,4] This method is efficient in monitoring transmission in areas in which onchocerciasis is hyperendemic or mesoendemic because the prevalence of infection in the vector population is usually high.[5,6] However, in the face of a successful control program, the prevalence of infection in the vector populations is drastically reduced. This is true both for programs relying on vector control[7] and for those using mass ivermectin distribution.[8] Thus, the classic dissection method to determine the prevalence of infection becomes progressively less efficient in areas where control has been successful.

Several years ago, a method based on the polymerase chain reaction (PCR) amplification of an *Onchocerca*-specific repeated DNA sequence (the O-150 PCR) was developed to identify *O. volvulus,* and to distinguish the blinding and non-blinding strains of the parasite.[9-11] This method has been successfully applied by the OCP to correct for the presence of animal filariae and non-blinding *O. volvulus* in calculations of the annual transmission potential for blinding onchocerciasis.[12] The O-150 PCR has also been shown to be capable of detecting a single infected fly in pools containing up to 100 flies.[13] Furthermore, a mathematical model has been developed to allow one to calculate prevalence of infection in the black fly population, based upon the size of the pools screened and on the percentage of negative pools found.[13] The assay and algorithm have been shown to accurately predict the prevalence of infection in simulated black fly populations prepared by mixing known numbers of experimentally infected flies and uninfected flies.[13] This pool screening technique could prove a useful adjunct to the classic dissection method because it may be capable of providing an early warning that transmission is occurring in a given area. If this is found, more elaborate studies can then be carried out in the endemic human population to better assess the situation. This could, in some cases, be the first step in the approach to the early detection of possible recrudescence.

For the PCR pool screening assay to be of practical use, it must first be demonstrated that the method is capable of accurately predicting the prevalence of infection in natural vector populations. It is particularly important to demonstrate that the method can be used in areas that have been subject to successful control measures, where infection rates are low. It would also be useful if the method could be used to distinguish infective from merely infected vector black flies. This is important since only the flies carrying the infective stage of the parasite are capable of contributing to transmission. This could be accomplished by restricting the analysis to the head capsules of the fly, in which only the infectious stage of the parasite is found.

In the following sections, we present data comparing the results obtained from dissection by trained technicians to

TABLE 1

Recovery of black fly heads using the freeze-sieve method

Collection point	Flies treated	Heads recovered	Percent yield
Bielikpong	1,547	1,485	96
Nabéré	900	840	93
Leraba Pont	902	846	94
Madina Diassa	4,058	3,979	98
N'zana	2,582	2,483	96

those obtained by PCR pool screening. We also present a method for rapidly separating the heads and bodies of *Simulium damnosum s. l*, thus allowing the assay to be applied only to the detection of flies carrying infectious parasites. The results suggest that the PCR pool screen assay may be a valuable addition to the tools for epidemiologic surveillance for *O. volvulus* infection.

MATERIALS AND METHODS

Collection of black fly specimens and purification of heads. To evaluate the PCR pool screening assay, *S. damnosum s. l.* were obtained from five collection points, following standard procedures.[3] These included one point in Ghana (Bielikpong on the Kulpawn River), two points in Burkina Faso (Nabéré on the Bougouriba River and Leraba Pont on the Leraba River), and two points in Mali (Madina Diassa and N'zana, both on the Baoulè River). A portion of the collected flies were dissected, and the number of flies carrying infective larvae in the head was noted. The parous rate of each fly population was also determined by examination of the ovaries of the dissected flies.[14] The larvae were preserved in a dried state suitable for subsequent PCR analysis, as previously described.[15] The species of each infective larva isolated from the dissected flies was determined using the O-150 PCR, followed by hybridization of the resulting PCR products to the *O. volvulus*-specific oligonucleotide probe OVS-2, as previously described.[16]

A second aliquot of flies from each collection point were preserved intact in isopropanol. Heads from the preserved flies were isolated using a modification of the method developed for the isolation of mosquito heads.[17] The preserved flies were rinsed twice in 95% ethanol and spread out on a watch glass. The flies were incubated at room temperature until the ethanol had evaporated. The flies were transferred to a 100-ml glass bottle and the bottle was placed in a Speedvac Cold trap (Savant Corporation, Farmington, NY) for 30

min. The heads were separated from the bodies by vigorous agitation of the bottle, which was accomplished by striking the bottle repeatedly against a rubber computer mouse pad. The separated heads and bodies were then placed in a 25-mesh brass sieve (Dual Manufacturing, Chicago, IL). Agitation of the sieve resulted in a purification of the heads from the bodies, as the heads passed through the sieve, while the bodies were retained.

Polymerase chain reaction analysis of purified black fly heads. The purified heads were divided into pools of a known size and DNA isolated from the pools as previously described.[13,16] The purified DNA was then used as a substrate in the O-150 PCR, and the resulting products were classified by hybridization to OVS-2, as previously described.[13,16] The prevalence of infection in the population was then determined using the PoolScreen® computer program. Poolscreen® provides estimates of the prevalence of infection in the vector population together with user selectable confidence intervals for these estimates, based upon the pool size, the number of pools examined, and the number of negative pools.[13] Poolscreen® is available free of charge from the corresponding author of this paper (TRU).

RESULTS

To distinguish infectious from infected flies in the pool screening assay, a method was developed to separate heads of *S. damnosum s. l.* from the thoraces and bodies. To estimate the efficiency of this method, the heads and bodies from each of the five collection points were separated *en masse,* and the total number of heads recovered from each point was determined. The results of these experiments are summarized in Table 1. The yield of heads ranged from 93% to 98%. Microscopic examination of the purified head preparation demonstrated that it consisted mostly of heads, together with a small amount of wing and leg fragments. No abdomens or thoraces were noted in the purified head preparations. These results suggested that the mass head purification procedure results in an almost quantitative recovery of heads from isopropanol-preserved black flies.

The heads recovered from each of the catch points were then used in the pool screen PCR assay, as described in the Materials and Methods. Table 2 presents the results comparing the predicted prevalence of *O. volvulus* infection at the five collection points as determined by dissection and by PCR screening of pools of heads. At three collection points, no *O. volvulus*-infected flies were found, either by dissection

TABLE 2

Calculated prevalences of *Onchocerca volvulus* infection in *Simulium damnosum s.l.* populations by dissection and pool screening

Collection point	Dissection					Pool screen					
	Number dissected	Parous rate (%)	Number infected	Uncorrected prevalence*†	Corrected prevalence*‡	Number examined	Pool size	Number of pools	Positive pools	Uncorrected prevalence*†	Corrected prevalence*‡
Bielikpong	10,792	61.5	20	1.1–**1.9**–2.9	1.8–**3.0**–4.6	1,480	20	74	3	0.4–**2.1**–6.0	0.7–**3.4**–10.0
Nebéré	1,020	65.3	0	0	0	840	20	42	0	0	0
Leraba Pont	1,224	65.2	1	0.02–**0.8**–4.5	0.03–**1.25**–6.9	840	20	42	2	0.3–**2.4**–8.8	0.4–**3.7**–13.4
Medina Diassa	1,633	64.1	0	0	0	3,950	50	79	0	0	0
N'zana	1,319	56.2	0	0	0	2,450	50	49	0	0	0

* Expressed as number of infectious per 1,000 flies. The center number in bold type represents the estimated prevalence, and the flanking values represent the 95% confidence interval of the prevalence estimate.
† Value not corrected for parous rate.
‡ Value corrected for parous rate.

or by pool screening. At the remaining two collection points, *O. volvulus*-infected flies were detected by both dissection and the pool screen assay. At Bielikpong, dissection predicted a prevalence of 0.19%, while the pool screen assay predicted a prevalence of 0.21%. Analysis of the confidence intervals surrounding these prevalence estimates demonstrated that they were not statistically different from one another (Table 2; $P > 0.5$). Similar results were obtained in the Leraba Pont collection point (Table 2; $P > 0.5$).

The level of infection as measured by dissection can be influenced by the overall age of the fly population. For example, the young adults will not have had a chance to take a blood meal, and will therefore will not have been exposed to *O. volvulus*. However, as the population ages, the majority of the flies would have become parous, and will therefore have had an opportunity to be exposed to infection. These differences in the age structure of the population at different sampling times and collection points can be compensated for by only considering the infection rate in the parous population.[18] For this reason, the results obtained by both dissection and pool screening were corrected for parous rate. For the dissected flies, this was accomplished by determining the prevalence of infection using the number of parous flies as the denominator. In the case of the pool screen assay, the percentage of parous flies as determined by dissection was used to estimate the number of parous flies in each pool, and this adjusted value was used as the pool size in the Pool-screen℗ algorithm.[13] Similar to what was found when the data were not corrected for parous rates, the prevalence of infection in the parous populations calculated by dissection and the pool screen method were not statistically different from one another (Table 2; $P > 0.5$).

DISCUSSION

The data presented demonstrate that the pool screen O-150 PCR assay produces estimates of infection prevalence in the vector population that are statistically indistinguishable from those obtained from the dissection of flies by trained technicians. After the cessation of control activities in the OCP area, the infectivity level of black flies is expected to be very low.[19,20] A similar situation is also likely to arise in areas subject to long-term ivermectin distribution.[8] The use of the PCR pool screening technique will make it possible to determine the level of transmission in a given area with a limited investment of human and material resources. This technique may thus be a valuable tool for the surveillance activities to be conducted in the OCP area following the cessation of active vector control in 2002. In addition, the ability to accurately measure infection rates in the vector population may prove useful in monitoring the effect of ivermectin-based onchocerciasis control programs (such as the Onchocerciasis Elimination Program in the Americas and the African Program for Onchocerciasis Control) on transmission. Furthermore, the pool screen PCR is an efficient means to screen large numbers of flies for the presence of *O. volvulus* larvae. It may thus prove useful in establishing that a given area is freed of *O. volvulus* transmission following successful control.

The capital costs involved in the PCR assay are considerable, requiring an expenditure of roughly $45,000 in spe-

cialized equipment. In contrast, capital costs of the classical dissection method are much less, roughly $2,000 for a dissecting microscope. However, following the initial capital expenditure, the marginal cost of the PCR method is roughly US $3.00 per 100 black flies assayed including supplies and labor, while the marginal cost of the classic dissection method is roughly US $15.00 per 100 black flies. This marginal cost advantage may prove to be particularly important in areas where control is effective, and it will be necessary to examine large numbers of flies to determine if transmission is still occurring. Furthermore, in West Africa, it will be necessary to carry out a PCR-based identification of larvae collected by classic dissection.[12] This is because *Onchocerca ochengi*, a bovine parasite, is sympatric with *O. volvulus* throughout most of West Africa. *Onchocerca ochengi* is also transmitted by *S. damnosum s.l.*, and *O. ochengi* and *O. volvulus* larvae are difficult or impossible to distinguish from one another using morphologic criteria.[21] Thus, in West Africa the capital expenditures associated with the PCR method will have to be borne whether or not the pool screen PCR or the classic dissection method is used.

Even outside of West Africa, where *O. ochengi* is not endemic and it will not be necessary to use a PCR assay to classify larvae detected by dissection,[22] the pool screen PCR may have cost advantages. Apart from the marginal cost advantage discussed earlier, costs associated with sample collection may prove to be less for the pool screen PCR. This is because the PCR assay can be applied to black flies preserved at ambient temperature stored in isopropanol. As described below, it may be possible to use volunteers from the local population to collect flies for the pool screen PCR. The flies can be stockpiled and then shipped without special handling to a central laboratory for analysis. Shipping can be coordinated between the peripheral and central health system infrastructures for minimal expense. In contrast, dissection requires freshly caught flies and must be performed at or near the capture site. This introduces costs associated with the travel of a trained technician to a location near each catching site, or the transportation of the freshly caught flies to a nearby laboratory. An additional cost related to the classic dissection method will be for the employment of fly catchers, who will also need to be paid salaries.

The results presented above also suggest that by correcting the pool size for parous rate, the pool screening technique can also give an accurate estimate of the infection rate in the parous fly population. Only parous flies will be infected with *O. volvulus*, since a fly must take a blood meal to be exposed to the parasite. The proportion of parous flies can vary widely depending on the age structure of the fly population, the climate, and even on the time of day that a collection takes place. Knowing the parous rate thus allows one to correct for these factors. For example, within the OCP, this corrected rate is currently expressed as the number of infective females per 1,000 parous females.[7,19,20] Unfortunately, the only current means of determining parity relies upon dissection of flies and examination of the ovaries for evidence of prior egg production.[14] Since this method requires a trained technician to dissect the flies, and the flies must be fresh, it cannot be used with samples collected by untrained volunteers.

Within the OCP area, the program has collected a large

amount of data recording parous rates at various collection points at various times throughout the year. These data may be exploited to develop collection regimens that maximize the proportion of parous flies collected, increasing the efficiency of the pool screen assay. Furthermore, the OCP data suggest that during the peak transmission period at the start of the rainy season, the parous rates fall within a relatively narrow range from year to year. Thus, within the OCP, historical data may be used to estimate parous rates during the periods of peak transmission. In the zones outside the OCP area where such data are not available, it may not be possible to correct the infection estimates for parous rate. In such situations, an uncorrected infectivity rate can be calculated, based on the number of infected flies for 1,000 females caught. However, it will be necessary to use the data as indicators of trends in infection prevalence, comparing results from the same season from year to year. This will allow one to account for the effect that fly migrations associated with intertropical front movements have on the age structure of the vector population, the parous rate, and consequently on the exposure of the vector population to *O. volvulus*.

For the pool screen assay to be an effective tool for the post-OCP era, it must be capable of being integrated into the existing health care infrastructure. Because of the technical complexity of the assay, it will be necessary to transport field-collected black flies to a centralized facility for analysis. In this regard, the potential of employing individuals in the endemic population to collect black flies deserves further scrutiny. Enlisting the endemic population will help to integrate the surveillance activities into the existing health care infrastructure. Employing residents in the villages as fly catchers may also be extremely cost-effective, especially when the catchers are designated by the community themselves. In addition, the initial experience with several of the countries in the OCP suggests that those in the health care infrastructure are willing to ensure the collection and transportation of such samples from the field to a central facility for further analysis. This suggests that it will be possible to integrate fly collection into the existing health care infrastructure in the countries of the OCP area.

Although the requirement for a central laboratory carries with it some obvious disadvantages, the use of a central facility has some advantages as well. By using a central laboratory, all data will be collected and analyzed in a central location. Such a centralized facility will therefore be capable of providing a picture of the situation throughout an entire area, while eliminating the inter-observer biases inherent in a decentralized monitoring structure. However, some important and practical challenges remain to be met before the PCR pool screening assay can be used as a routine tool for onchocerciasis surveillance in the OCP area. These include developing a protocol for the timely transportation of the samples to the laboratory, the management of the central laboratory in the post-OCP era, and the integration of this type of surveillance procedure into the activities of the decentralized health structures of the countries that are now part of the OCP. Discussions to resolve these remaining problems are under way with the various partners in health development, in the context of planning for the phasing out of the OCP. These discussions are expected to result in establishment of concrete structures and policies for the trans-

fer of the residual activities of the OCP before the program ends in 2002.

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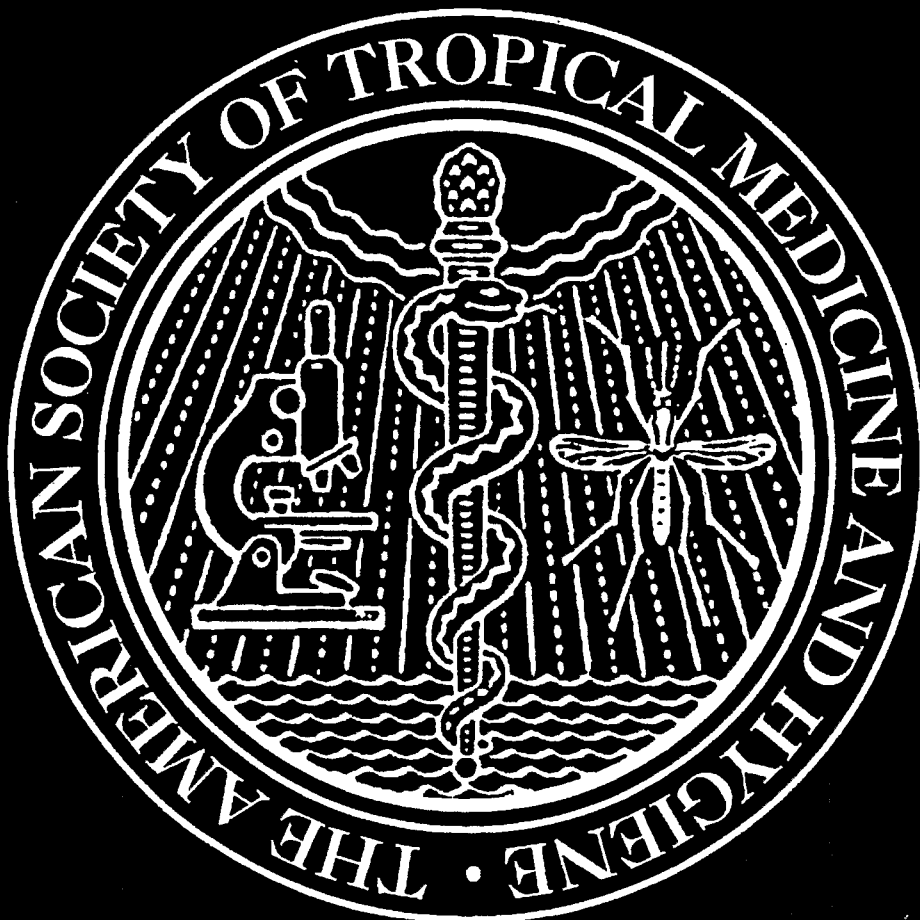
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