INTRODUCTION

Onchocerciasis, or river blindness, is the target of three large international disease control programs: the Onchocerciasis Control Program (OCP) in West Africa, the African Program for Onchocerciasis Control (APOC), and the Onchocerciasis Elimination Program for the Americas (OEPA). These programs differ in their approaches and in their progress toward their final goal of eliminating onchocerciasis as a public health problem. However, both the OCP and OEPA share a common strategic goal: to eliminate transmission of the causative agent of the disease, the filarial parasite Onchocerca volvulus. The thesis underlying this goal is that if transmission can be effectively suppressed for a suitable period of time, the reproductive rate of the parasite will be brought below the minimum replacement rate, and the infection will die out. Evidence that this approach can succeed is provided by the success of the OCP, which has succeeded after almost 20 years of transmission suppression (primarily through vector control) in eliminating onchocerciasis as a public health problem.

If one is to use a strategy of suppressing transmission using chemotherapy to eliminate human onchocerciasis, monitoring the level of transmission is necessary. This is required to demonstrate that the control strategy being applied is effective and to ensure that once control activities are in place, transmission has been brought to the point where the parasite population can no longer sustain itself. O. volvulus transmission has historically been measured through dissection of vector black flies. This is an efficient method of monitoring transmission in areas not subject to control, because the prevalence of infection in the vector population is usually high. However, in the face of a successful control program, the prevalence of infection in the vector populations is drastically reduced. Thus, the classic dissection method to determine the prevalence of infection becomes progressively less efficient in areas where control has succeeded. 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 nonblinding strains of the parasite. The O-150 PCR can detect a single infected fly in pools containing up to 100 flies. Furthermore, a mathematical model has been developed to allow one to calculate the prevalence of infection in the black fly population based on the size of the pools screened and on the percentage of negative pools found. In addition, a method for the mass separation of heads and bodies of vector black flies has recently been reported. This allows one to obtain separate estimates of the prevalence of parasites in the head capsule (representing the L3 or vertebrate infectious stage) and bodies (representing the earlier developmental stages, i.e., the L1 and L2 larvae). In field-based studies in Africa and Mexico, the O-150 pool screen PCR assay was found to produce estimates of the prevalence of infected and infective flies in the vector population that were indistinguishable from those obtained by traditional dissection. These results suggested that the O-150 PCR pool screen assay might be a useful tool to monitor transmission in areas where successful control measures were in place and the level of infection in the vector population was low.

Ecuador is one of the six countries in the Americas endemic for onchocerciasis and therefore is a member of the regional initiative for elimination of the disease. Onchocerciasis is endemic in three river basins in Ecuador: the Rio Santiago, the Rio Cayapas, and the Rio Canande. The vectors of O. volvulus in Ecuador include Simulium exiguum, a highly efficient limitation-type vector which is comparable to the African forest species in terms of the percentage of flies developing infective stage larvae and the number of larvae per infected fly, and S. quadrivittatum, a species that is thought to be a less-efficient vector. Mectizan™-based elimination efforts have been under way in Ecuador for the past decade, beginning in 1990 with the Rio Santiago area, where by 1997 transmission was thought to have been brought below the level necessary to maintain the infection. Here we report the results of a large-scale project to assess
O. volvulus transmission in Ecuador, using the O-150 pool screen PCR as a monitoring tool. This assay was used to examine in excess of 100,000 flies collected at seven sentinel communities in the three endemic river basins of onchocerciasis in Ecuador during the 2000 transmission season. The data demonstrate a sustained interruption of transmission in the Rio Santiago area since 1997, significant progress toward elimination of transmission in the other river basins, and the usefulness of the O-150 pool screen PCR to efficiently monitor transmission in areas where Mectizan™-based elimination programs are being implemented.

MATERIALS AND METHODS

Black fly collection. Since 1991, Ecuador has been part of the multinational, multiagency OEPA partnership, formed to eliminate onchocerciasis through semiannual treatments with Mectizan™. OEPA guidelines for monitoring and evaluation of Mectizan™ programs include the designation of sentinel communities (mostly hyperendemic) within endemic areas that are subject to in-depth evaluation at about 5-year intervals. Parasitologic indices (skin snip prevalence, community microfilarial skin density, ocular infection) and entomologic indices (prevalence of infective stage larvae in vector populations and monthly and annual transmission potentials) are determined as part of these in-depth evaluations. To the extent that sentinel communities represent all communities within a focus, the in-depth evaluations measure program achievement toward the ultimate goal of eliminating onchocerciasis. The communities included in this study represented the designated sentinel communities for the Rio Santiago, Rio Cayapas, and Rio Caninde foci in Esmeraldas Province, Ecuador.

Flies were collected following standard procedures. Collections were carried out in the transmission seasons of March–June 1996 and April–July 2000. The flies collected in 2000 were separated according to species, divided into aliquots of 50 flies each, and preserved in isopropanol at room temperature. Flies collected in 1996 were not separated according to species but were handled similarly, except that they were divided into pools of 25 each.

Material preparation and O-150 amplification. Isopropanol-preserved pools were rinsed twice in 95% ethanol, air-dried briefly, and placed into 15-mL conical centrifuge tubes. The flies were then placed in liquid nitrogen overnight. In the following day, the flies were homogenized in a solution of 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% SDS, 100 μg/mL proteinase K and 3 μg/mL salmon sperm DNA, and incubated at 56°C for one hour. Dithiothreitol was added to a final concentration of 10 mM and the homogenate heated to 100°C for 30 minutes. The samples were subjected to two freeze-thaw cycles, followed by extraction with phenol and chloroform (1/1 v/v). DNA was purified from the aqueous layer by two cycles of adsorption to silica powder, as previously described. The purified DNA was eluted into a final volume of 50 μL of 10mM Tris-HCl (pH 8.0) 1 mM EDTA.

PCR amplifications were carried out in a total volume of 50 μL in a solution consisting of 60 mM Tris-Hcl (pH 9.0) 15 mM (NH₄)2SO₄, 2 mM MgCl₂, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 0.5 μM of each primer, 2.5 units of Taq polymerase (Roche Diagnostics, Indianapolis, IN), and 2.5 μL of template DNA. The sequence of the primers used was as follows: 5’ GATTTYTCCGRCGAANARCGC 3’ and 5’ B-GCNRT-RTAAATNTGNAATTCT 3’; where N = A, G, C, or T; Y = C or T; R = A or G; and B = biotin. Cycling conditions consisted of five cycles of 1 minute at 94°C, 2 minutes at 37°C, and 30 seconds at 72°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 37°C, and 30 seconds at 72°C. The reaction was completed by incubation at 72°C for six minutes. Reactions were run in sets of 96, which included negative controls and two positive controls.

PCR product identification and data analysis. PCR amplification products were detected by PCR enzyme-linked immunosorbsent assay (ELISA), essentially as previously described. In brief, 96-well Immulon 2 plates (Dynex, Chantilly, VA) were coated overnight with 1 μg/mL strepavidin in 50 mM NaHCO₃ and 2 mM Na₂CO₃. After coating, the plates were washed six times in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20 (TBST). A total of 10 μL of each PCR product was placed in each well and combined with 40 μL of a solution consisting of 4.8X SSPE, 1X Denhardt’s solution, 0.1% N-lauryl sarcosine, and 0.02% SDS (HB). The plates were incubated at room temperature for 30 minutes and washed six times with TBST. A total of 100 μL of 1N NaOH was added to each well, and the plate was incubated for 1 minute at room temperature and washed six times with TBST. A total of 50 μL of a solution of 50 ng/mL of the detection oligonucleotide OVS2-FL in HB was added to each well and the plate incubated at 42°C for 15 minutes and washed six times with TBST. The sequence of OVS2-FL was 5’ AATCTCAAAAAACGGGTACATA-fl 3’ where fl = fluorescein. A total of 100 μL of 1X SSPF and 0.1% SDS prewarmed to 42°C was added to each well, and the plate was incubated at 42°C for 10 minutes. The plate was washed six times with TBST and 50 μL of a 1/10,000 dilution of antifluorescein Fab fragment - AP conjugate (Roche Diagnostics) in 0.4M NaCl, 0.1M Tris-HCl (pH 7.5) 0.5% (w/v) bovine serum albumin was added to each well. The plate was incubated at 37°C for 15 minutes and washed six times with TBST. Bound probe was detected using the Amplify AP substrate kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Any samples giving an OD value above the mean plus three standard deviations of the 10 negative control wells was considered provisionally positive. Provisionally positive DNA samples were subjected to a second independent PCR amplification and PCR-ELISA assay. Provisionally positive samples that scored positive in the second PCR amplification were scored confirmed.

Statistical analysis. The prevalence of infection in the vector population and confidence intervals surrounding this estimate were calculated as previously described, using the algorithms contained in Poolscreen v1.1. Poolscreen uses a statistical model based on the observation that given the pool size and the probability of infection of an individual black fly, the probability that a pool is negative is easily calculated. Each pool represents an independent trial with outcome after testing of positive or negative. The number of positive pools observed in a set of m independent trials is well known to
The prevalence of infected flies as estimated by microscopic examination of skin snips ranging from 53–96% (Table 1). At the time of the infection, with infection prevalence 1 year of first treatment round, the overall prevalence of infected flies dropped roughly 16-fold (from 176/10,000 flies to 10.7/10,000 flies) from 1996 to 2000. Similarly, the prevalence of infective flies (calculated from the infection rate in pools of heads, representing flies carrying infective stage larvae) and the overall prevalence of infection (calculated from the overall number of positives in either the head or body pools). The results of these analyses are summarized in Table 2. None of the pools examined from the three sites in the Rio Santiago basin contained detectable O. volvulus DNA. In contrast, evidence for O. volvulus transmission was detected in both the Rio Cayapas and Rio Canande basins. The rate of infected flies (calculated from the body pools) ranged from 2.1/10,000 flies (in S. exiguum collected in Naranjal on the Rio Canande) to 34.8/10,000 flies (in S. quadrivittatum collected in San Miguel on the Rio Cayapas). The prevalence of infective flies was lower than the prevalence of infected flies, ranging from 0.5/10,000 flies (in S. exiguum at Naranjal) to 15.6/10,000 flies (at El Tigre on the Rio Cayapas).

As expected, the pool screen PCR data revealed a dramatic effect of Mectizan™ distribution on transmission (Table 2). In San Miguel, the overall prevalence of infected flies dropped roughly 16-fold (from 176/10,000 flies to 10.7/10,000 flies) from 1996 to 2000. Similarly, the prevalence of infective flies dropped roughly 60-fold (from 65/10,000 flies to 1.1/10,000 flies). Large decreases in both the infected and infective rate also were noted in the flies collected at El Tigre (Table 2).

Vectorial capacity results from a combination of factors, including biting rate, host preference, the efficiency of parasite development in the insect vector (vector competence), parasite developmental time in the vector, and the average lifespan of the insect. It was therefore possible that S. exiguum and S. quadrivittatum might exhibit differences in their

### RESULTS

Flies were collected from seven sentinel communities in the three river basins endemic for O. volvulus in Esmeraldas Province, Ecuador. Esmeraldas is located in the north of Ecuador along the Pacific coast. The onchocerciasis endemic areas are located in the interior of the province. Demographic statistics of the seven sentinel communities relevant to the current study are presented in Table 1. Before the beginning of Mectizan™ distribution, all seven communities were hyperendemic for O. volvulus infection, with infection prevalences (as estimated by microscopic examination of skin snips) ranging from 53–96% (Table 1). At the time of the in-depth evaluation conducted in 2000, all communities had received 6–14 rounds of Mectizan™ treatment at annual or semiannual intervals.

A total of 5,700 flies were obtained before the beginning of Mectizan™ distribution at two sites (San Miguel and El Tigre) in 1996, and were not divided according to species. In contrast, flies were obtained from all seven sites in 2000, representing a time period of four to ten years after the start of Mectizan™ distribution, depending on the site.

As previously reported, two species of black fly serve as vectors for O. volvulus in Ecuador: S. exiguum and S. quadrivittatum. Both species were found at all seven collection sites. However, the proportion of the two species varied. S. exiguum predominated at sites along the Rio Cayapas (Corriente Grande, El Tigre, and San Miguel) and the Rio Canande (Naranjal) while S. quadrivittatum represented the majority of flies collected at the sites on the Rio Santiago (Playa de Oro, Angostura, and Guayabal) (Table 2). In total, 102,200 flies were collected during 2000 (66,250 S. exiguum and 35,950 S. quadrivittatum).

Collecting flies were separated into aliquots according to month, site of collection, and species, and the aliquots were divided into pools. For the precontrol flies collected in 1996, the pools contained 25 flies each, while for the postcontrol flies collected in 2000, pools of 50 individuals were constructed. The heads and bodies in each pool were separated, DNA prepared from each separated head and body pool, and each DNA sample tested for the presence of O. volvulus DNA using the O-150 PCR assay. The prevalence of infection in each group of pools was then calculated using the Poolscreen program. In calculating infection rates in the vector population, three analyses were performed. These included the prevalence of infected flies (calculated from the infection rate in pools of bodies, representing flies carrying developing stages of the parasite), the prevalence of infective flies (calculated from the infection rate in pools of heads, representing flies carrying infective stage larvae), and the overall prevalence of infection (calculated from the overall number of positives in either the head or body pools). The results of these analyses are summarized in Table 2. None of the pools examined from the three sites in the Rio Santiago basin contained detectable O. volvulus DNA. In contrast, evidence for O. volvulus transmission was detected in both the Rio Cayapas and Rio Canande basins. The rate of infected flies (calculated from the body pools) ranged from 2.1/10,000 flies (in S. exiguum collected in Naranjal on the Rio Canande) to 34.8/10,000 flies (in S. quadrivittatum collected in San Miguel on the Rio Cayapas). The prevalence of infective flies was lower than the prevalence of infected flies, ranging from 0.5/10,000 flies (in S. exiguum at Naranjal) to 15.6/10,000 flies (at El Tigre on the Rio Cayapas).

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### Table 1

Demographic characteristics of sentinel communities

<table>
<thead>
<tr>
<th>River basin</th>
<th>Community</th>
<th>Population</th>
<th>Pretreatment infection prevalence</th>
<th>Year of first treatment</th>
<th>Number of treatment rounds</th>
<th>Mean coverage²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rio Cayapas</td>
<td>El Tigre</td>
<td>118</td>
<td>94.2</td>
<td>1996</td>
<td>6</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>San Miguel</td>
<td>181</td>
<td>96.3</td>
<td>1996</td>
<td>6</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Corriente Grande</td>
<td>179</td>
<td>96.3</td>
<td>1991</td>
<td>14</td>
<td>82</td>
</tr>
<tr>
<td>Rio Canande</td>
<td>Naranjal</td>
<td>447</td>
<td>53.3</td>
<td>1991</td>
<td>12</td>
<td>77</td>
</tr>
<tr>
<td>Rio Santiago</td>
<td>Playa de Oro</td>
<td>243</td>
<td>66.6</td>
<td>1990</td>
<td>12</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Angostura</td>
<td>78</td>
<td>80.6</td>
<td>1991</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Guayabal</td>
<td>130</td>
<td>63.6</td>
<td>1990</td>
<td>12</td>
<td>85</td>
</tr>
</tbody>
</table>

¹ As estimated by microscopic examination of skin snips
² Mean percentage of eligible population receiving treatment
vectorial capacities that would be reflected in the fly collections examined here. To test this hypothesis, the prevalence of infected and infective *S. exiguum* and *S. quadrivittatum* were analyzed from the four collection points where the data presented in Table 2 suggested that *O. volvulus* transmission was still occurring. The results of this analysis is summarized in Table 3. The prevalence of infected flies was significantly higher in *S. quadrivittatum* than in *S. exiguum* (21.5/10,000 flies versus 6.2/10,000 flies, *P* < 0.01; fiducial confidence interval). In contrast, the prevalence of infective flies was higher in *S. exiguum* than in *S. quadrivittatum*, although this difference was not statistically significant (*P* > 0.05; fiducial confidence interval). Thus, the ratio of the prevalence of infected *S. quadrivittatum* flies to infective *S. quadrivittatum* flies was roughly 20/1 while the corresponding ratio for *S. exiguum* was roughly 2/1.

The number of flies of each species collected and the overall infection rate during each month for the collection points where *O. volvulus* was detected are summarized in Figure 1. The overall number of flies of each species collected and the prevalence of infection in each species did not vary significantly from month to month at the collection points in the Rio Cayapas basin (Figure 1). In Naranjal, in the Rio Canande basin, infected flies were seen only in June 2000, while the small number of *S. quadrivittatum* collected at this site was obtained only during May (Figure 1).

**DISCUSSION**

Entomologic evaluations present several advantages as a means of monitoring the effect of onchocerciasis control programs. Perhaps most important, measurement of transmission levels is the most direct method of assessing a program’s progress toward the major strategic goal of preventing transmission. Unfortunately, in areas where control is successful, the prevalence of infected flies is quite low, meaning that a very large number of flies must be examined to accurately measure transmission levels. Pool screen PCR-based methods represent a way of efficiently examining the large numbers of flies necessary to accomplish this goal. In previous laboratory and field-based pilot studies, the O-150 pool screen PCR has been shown to produce estimates of infection prevalence that were not statistically different from those obtained from traditional dissection. The data presented above represent the first large-scale countrywide application of this method by an onchocerciasis control program.

These data support the findings of previous pilot studies demonstrating that Mectizan™ treatment can dramatically decrease transmission of *O. volvulus*. In these studies, multiple treatments with Mectizan™ have been shown to reduce transmission of *O. volvulus* by anywhere from 92–95%. In both El Tigre and San Miguel, similar decreases in the prevalence of both infected and infective flies were seen after six semiannual treatments with Mectizan™. This suggests that in countries with effective Mectizan™-based control programs, transmission can be dramatically reduced, in line with predictions from the previous field trials.
None of the 29,900 flies examined from the three collection sites in the Rio Santiago basin were found to contain *O. volvulus*. The upper boundary for the 95% confidence interval for the prevalence of infection in a fly population when 29,900 flies are examined and all are found to be negative (as calculated by Poolscreen) is 0.6 per 10,000 flies. In a study published in 1997, Guderian and co-workers reported that the vector infection prevalence in the Rio Santiago basin had been reduced by Mectizan™ treatment over an 84-month period to roughly 8/10,000 flies, from a precontrol level of 110/10,000 flies. Furthermore, no new infections in the human population were detected from 1994 onward. Based on these results, the authors suggested that *O. volvulus* transmission in the Rio Santiago basin had been brought below the level needed to sustain new infections. The fact that we could not detect any infected flies during this study suggests that the downward trend in transmission in the Rio Santiago area noted by Guderian in 1997 had continued, and that infection of the vector population may have ceased by 2000.

Previous studies have noted that the percentage of *O. volvulus* microfilaria developing to infective larvae is greater in black flies lacking a cibarial armature when compared to flies that contain this structure. This effect is believed to be due to damage to ingested microfilaria in black flies containing a cibarial armature. Thus, black flies lacking a cibarial armature are generally more-competent vectors for *O. volvulus* than those that contain a ciberal armature. The data presented above support this, as the proportion of infected flies to infective flies was 2/1 in *S. exiguum*, which lacks a ciberal armature, while it was 20/1 in *S. quadrivittatum*, which contains this structure. However, vectorial capacity of a given species is a complex function that involves a plethora of factors, of which vector competence is only one. In this regard, it is interesting to note that although *S. quadrivittatum* appears to be a less-competent vector than *S. exiguum*, the prevalence of infection in *S. quadrivittatum* was significantly higher than in *S. exiguum*. Thus, although *S. quadrivittatum* was a less-competent host for larval development than *S. exiguum*, the prevalence of infective flies in the two species was not statistically different, suggesting that *S. quadrivittatum* is playing an important role in maintaining *O. volvulus* transmission in areas of Ecuador where transmission has not been completely interrupted.

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REFERENCES