

POLYMERASE CHAIN REACTION MONITORING OF TRANSMISSION OF *ONCHOCERCA VOLVULUS* IN TWO ENDEMIC STATES IN MEXICO

MARIO A. RODRÍGUEZ-PÉREZ, BRUCE G. LILLEY, ALFREDO DOMÍNGUEZ-VÁZQUEZ,
RAMÓN SEGURA-ARENAS, CRISTIAN LIZARAZO-ORTEGA, ALBERTO MENDOZA-HERRERA,
FILIBERTO REYES-VILLANUEVA, AND THOMAS R. UNNASCH

Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico; Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, Alabama; Dirección de Servicios de Salud, Instituto de Salud del estado de Chiapas, Tuxtla Gutiérrez, Chiapas, Mexico; Programa de Oncocercosis, Secretaría de Salud de Oaxaca, Oaxaca, Mexico; Laboratorio de Entomología Médica, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL Mexico

Abstract. To investigate the impact of mass ivermectin treatments in Mexico on *Onchocerca volvulus* transmission, entomologic surveys were carried out in the two endemic states of Oaxaca and Chiapas. The data suggest that substantial progress towards the goal of elimination has been made. A comparison pre- and post-ivermectin data from a community in Southern Chiapas showed a 97% decrease in seasonal transmission potential, but some level of polymerase chain reaction positivity was still detectable. In other communities from northern Chiapas and Oaxaca where there are no baseline data, there was an absence or near absence of infective flies. Residual transmission was not evenly distributed because differences were seen in the infection and infective rates from different catch points. These findings suggest that while substantial progress towards elimination has been made in Mexico, it may be necessary to modify ivermectin distribution strategies to eliminate transmission in areas where transmission persists.

INTRODUCTION

Onchocerciasis, or river blindness, still poses a threat to public health in Africa and Latin America. It is caused by infection with the filarial parasite *Onchocerca volvulus*. In Mexico, *O. volvulus* occurs in three endemic foci located in Oaxaca and in southern and northern Chiapas. This represents an area of roughly 16,932 km² with a population of approximately 192,000 inhabitants, of which 25,000 are infected with *O. volvulus*.^{1–3}

Onchocerciasis is being combated in Latin America by the Onchocerciasis Elimination Program in the Americas (OEPA) whose major goal is to eliminate severe pathologic manifestations of the disease and to reduce morbidity through mass distribution of ivermectin, a microfilaricidal drug.² There is also hope that if the human microfilarial load is reduced by mass ivermectin treatment to a level that is below what is necessary to maintain transmission, transmission may be interrupted and the infection eventually eliminated. Unfortunately, both the minimum reproductive rate and the level of transmission necessary to maintain the minimum reproductive rate for *O. volvulus* remain to be precisely determined. In the absence of this essential information, the World Health Organization has developed a series of criteria for certifying that an area is free of onchocerciasis.⁴ These criteria are based upon a demonstration that transmission and infection incidence have dropped below a certain specified value and remain below this value for a period of seven years.⁴ Two different measures of transmission suppression are currently used. In areas where pre-treatment data are available, suppression of infectivity is defined as a 99% reduction in transmission from pre-treatment rates. In areas where pre-treatment data are not available, suppression of infectivity has been set at a level of no more than one in 10,000 flies carrying infective larvae.⁴ Suppression of transmission has been achieved through ivermectin distribution in some areas in Latin America,^{5–7} and with vector control in Africa.⁸ However, progress towards interruption of transmission in Mexico has been slower, despite multiple semi-annual treatments with ivermectin.^{3,9–11}

In the Mexico-Guatemala region, onchocerciasis is associated with coffee plantations, and the seasonal third-stage larvae (L3) transmission peak occurs in the late dry season in association with the presence of old, multiparous *Simulium ochraceum* sensu lato females.^{12–14} It has been suggested that the origin of the onchocerciasis focus in southern Chiapas was a consequence of the migration of coffee plantation laborers from Guatemala, and that the northern Chiapas focus was established as a result of annual visits of northern residents to the southern Chiapas focus for the coffee harvest.^{14,15} If this was the case, the northern Chiapas endemic focus may be made up entirely of imported cases of onchocerciasis. It is not known if autochthonous transmission exists at this focus.

The Oaxaca endemic focus does not seem to have any epidemiologic link with the other foci in Chiapas and Guatemala. Its origin may be due in part by the human movements to the south to perform religious pilgrimages. Parasitologic findings showed that the transmission in Oaxaca may be suppressed.¹⁶ However, this finding needs to be confirmed by entomologic studies to ascertain that effective ivermectin coverage has indeed been able to suppress transmission and that this may eventually reduce reproduction in the parasite population below the minimum reproductive rate breakpoint.

Detection of natural infection levels in simuliid populations classically has been accomplished through the dissection of wild caught flies. This technique is capable of estimating parity rates and transmission levels; however, in the face of an effective control program, the infection prevalence in fly populations becomes drastically reduced. This means that a very large number of flies must be examined to accurately measure transmission levels, limiting the value of dissection as a tool for estimating infection rates in the vector population. Polymerase chain reaction (PCR) assays for detecting *O. volvulus* larvae in flies have been developed over the past decade. These are based on the amplification of an *Onchocerca* repeated sequence gene family (O-150), followed by hybridization of the amplified products with an *O. volvulus* species-specific DNA probe.^{17–19} The PCR data generated by screening pools of flies can then be analyzed to calculate the

proportion of flies infected, and the mean larval load per fly can be further estimated from these data.^{11,20,21} This approach has been validated in Mexico in a comparison study between a PCR and the dissection in which both methods produced statistically similar estimates of the prevalence and infection intensity in a fly local population.¹¹ The PCR pool screen method has also recently been used in a large-scale study to assess infection prevalence in *S. exiguum* and *S. quadrivittatum* from the Ecuadorian endemic areas subject to in-depth entomologic evaluations.⁷

Here, we report data arising from a large-scale study to evaluate *O. volvulus* transmission by an O-150 pool screen PCR in seven sentinel communities in the three onchocerciasis endemic foci of Mexico.^{2,3} The procedure used here may be applicable to other control programs targeting filarial infections transmitted by *Simulium* or other arthropod vectors.

MATERIALS AND METHODS

Characteristics of the sentinel communities. Mexico began ivermectin distribution for onchocerciasis in 1989, treating only symptomatic individuals. In 1991, the program was expanded to include all eligible individuals in selected communities, most of which were hyperendemic for *O. volvulus* infection. Since 1995, distribution of ivermectin has been scheduled semi-annually for all at risk communities. At the time of the present study, the communities had received 17 rounds of ivermectin treatment. Average coverage with ivermectin of all eligible individuals in 2001 was 88%, which represented the distribution of approximately 300,000 oral doses of ivermectin.³

Mexico is a member of OEPA and follows the recommendations suggested by OEPAs expert committee. Committee guidelines include the designation of sentinel hyperendemic communities for each endemic focus and an in-depth epidemiologic evaluation of these sentinel communities at roughly four year intervals. An in-depth epidemiologic evaluation includes determination of the ivermectin coverage of the eligible population, the prevalence and intensity of skin microfilarial community load, the prevalence of microfilariae in the anterior chamber of the eye, the prevalence of infection in insect vectors, the annual transmission potentials, the seroprevalence and the positive seroconversion in the population ≤ 5 years of age (i.e., those born since the previous evaluation). In this study, the four sentinel communities of Oaxaca (La Chichina, La Esperanza, Santiago Lalopa, and Santiago Teotlaxco) and two of the six sentinel communities of southern Chiapas (Morelos and Ampliación Malvinas) were included. Sentinel communities for the northern Chiapas endemic focus have not been officially identified. Altagracia in northern Chiapas was selected as a study site based on the recommendation of local health officials. The affected ethnic population in Oaxaca and northern Chiapas are indigenous peoples, while the affected population in southern Chiapas are of mixed heritage (Table 1). The most important economic activity in all foci is coffee production.

Fly collection and processing. *Simulium ochraceum* s.l. were collected using the human landing-bait method. Volunteers for this purpose were selected from the endemic communities, and performed in teams of two, representing a bait and a collector. All participants received ivermectin treatment before beginning fly collection activities. Ethical and

TABLE 1
Demographic characteristics of the study communities in Mexico

Focus/community	Population	Predominant ethnic group	Skin microfilaria prevalence*
Oaxaca			
Santiago Teotlaxco	456	Indigenous, mainly Zapoteco and Chinanteco	7%
Santiago Lalopa	562		
La Esperanza	236		
La Chichina	368		
Total	1,622		
Southern Chiapas			
Morelos	382	Mixed heritage	16%
Ampliación Malvinas	234		
Total	616		
Northern Chiapas			
Altagracia	236	Indigenous, mainly Chamula, Tzotzil, and Zoque	ND
Grand total	2,474		

* As estimated by skin snip. ND = not determined.

biosafety procedures followed those of the corresponding committees of the National Institute of Public Health (Mexican Health Ministry, Cuernavaca, Morelos, Mexico). Insect collections were carried out as previously described.^{9,11,13} Daily entomologic sampling began at 11:00 AM and ended at 4:50 PM, which represents the diurnal period with the highest proportion of parous hematophagous flies.²² Collections consisted of 50-minute sampling units followed by 10-minute breaks. The collection of insects was conducted simultaneously in two sites for each community, one in a nearby coffee plantation and the other within the community, during the transmission season from February to May 2001. To determine the *S. ochraceum* s.l. parity rate in each community, daily collections and dissection of *S. ochraceum* s.l. were performed according to the method of Cupp and Collins.²³ The simuliids were collected before procuring a blood meal. Therefore, what we refer to as biting rates will be more accurately classified as landing rates. The latter may overestimate the former because feeding success was not measured.²⁴

Following collection, flies were preserved in isopropanol and stored at room temperature. Preserved flies were identified to the species level, and the few flies found to contain traces of a blood meal were discarded. *Simulium ochraceum* s.l. flies were separated into individual pools each containing 50 flies each, rinsed three times in 95% ethanol, air-dried briefly, and placed into individual 2.0-mL polypropylene cryotubes. Five of these cryotubes at a time were transferred to a conical 50-mL polypropylene centrifuge tube and the tube placed in liquid nitrogen for 30 minutes. The tubes were removed from the liquid nitrogen and subjected to vigorous agitation to separate the heads and bodies. The heads were then purified from the bodies by passage through a 25-mesh sieve. The heads and bodies were collected into separate 1.5-mL microcentrifuge tubes and processed separately.⁷

Purification of DNA. The separated pools of heads and bodies were homogenized in a buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 100 μ g/mL of proteinase K, and 3 μ g/mL of salmon sperm DNA. The homogenates were incubated at 55°C for one hour to digest the included protein and boiled in the presence of 10 mM dithiothreitol to disrupt the parasite

cuticle. Boiling was followed by a series of freeze-thaw steps to release the DNA parasite from the parasites. The DNA was then purified from the individual preparations by two cycles of extraction with 1:1 (v/v) phenol-chloroform, followed by one extraction with chloroform. The aqueous layer of this solution was then transferred to a well of a deep well microtiter plate. The DNA was purified in the deep well microtiter plate by two rounds of glass absorption as previously described.¹¹ Purified DNA was then eluted with 50 μ L of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and stored at -80°C .

Analysis by the O-150 PCR. All PCRs were carried out in sets of 84 samples, in rows B-H of a PCR microtiter plate. A volume of 2.5 μ L of the purified genomic DNA was used as a template for the PCR amplifications carried out in a total volume of 50 μ L containing 0.5 μ M of O-150 primer (5'-GATTYTTCCGRCGAANARCGC-3') and 0.5 μ M of biotinylated O-150 primer (5'-B-GCNRTRTAAATNTG-NAAATTC-3', where B = biotin; N = A, G, C, or T; Y = C or T; and R = A or G). Reaction mixtures also contained 60 mM Tris HCl, pH 9.0, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 0.2 mM each of dATP, dCTP, dGTP and dTTP, and 2.5 units of *Taq* polymerase (Roche Diagnostics, Indianapolis, IN). Cycling conditions consisted of five cycles of one minute at 94°C , two 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. Row A was reserved for 10 negative controls and two positive controls. One positive control contained the minimal amount of positive control DNA found to be consistently detected by the PCR amplification conditions, as determined by an initial titration study. This control was carried out to ensure that all of the reaction sets were operating at peak efficiency. The second positive control contained the same minimal amount of positive control DNA mixed with 2.5 μ L of a DNA preparation from a pool that tested negative in a prior set of reactions. This control ensured that no inhibitors were present in the fly DNA preparations.

The PCR amplification products were detected by PCR enzyme-linked immunosorbent assay (ELISA) essentially as previously described.^{7,25,26} Briefly, 10% of each PCR amplification reaction (5 μ L) was bound to a streptavidin (1 μ g/mL)-coated ELISA plate, and the DNA strands denatured by treatment with alkali. The bound PCR fragments were then hybridized to a fluorescein-labeled *O. volvulus*-specific oligonucleotide probe (OVS2: 5'-AATCTCAAAA-AACGGGTACATA-FL-3'), and the bound probe detected with an alkaline phosphatase-labeled anti-fluorescein labeled antibody (fragment FA; Roche Diagnostics). Bound antibody was detected using the ELISA amplification reagent kit from Invitrogen/BRL (Carlsbad, CA) following the manufacturer's instructions. Color development was stopped by the addition of sulfuric acid, and the plates read in an ELISA plate reader set at 450 nm. The cut-off for classifying a sample as putatively positive was set at the mean of the 10 internal negative controls plus three standard deviations, or 0.1, whichever was greater. A second independent PCR and ELISA were then carried out on the putatively positive samples. Only putative samples classified as positive in both independent reactions were scored as confirmed positive.

Data analysis. Five entomologic parameters were selected for analysis in this study. These were as follows.

Infection rate in the parous vector population. This was calculated from the proportion of body pools positive in the PCR assay, and expressed per 10,000 flies.

Infective rate in the parous vector population. This was estimated from the proportion of head pools positive in the PCR assay and expressed per 10,000 flies. The Poolscreen[®] computer program²¹ was used to estimate both proportions and the 95% confidence intervals (CIs) surrounding these parameters. To reflect the proportion of parous flies in the population assayed by PCR, the pool size in the Poolscreen[®] algorithm²¹ was adjusted.²⁷ For example, for a parous rate of 80% and a pool size of 50 flies, the infection rate in the parous population was corrected by entering a pool size of 40 in the Poolscreen[®] program, reflecting the average number of parous flies in each pool.²⁷

Larvae per parous fly. This value was derived from the observed proportion of positive parous flies with any *O. volvulus* larval stage. This was calculated by determining the number of flies carrying larvae in either the head or body divided by the total number of parous females tested. This estimate takes into account a given degree of larval aggregation, with $k = 0.273$ assumed to be a known parameter for *S. ochraceum* s.l.²⁰

Monthly biting rate (MBR). The MBR was obtained from the number of flies caught multiplied by the number of days in month and divided by the number of catching days, as described by Walsh and others.²⁸ The MBR was divided by two (the number of sampling sites in each community) to obtain the mean MBR per person. The MBR of parous flies was obtained by multiplying the overall MBR by the proportion of parous flies found at each community. The standard deviation of the MBR of parous flies was calculated as the square root of the variance of the product of the MBR of parous flies times proportion of parous flies, using the method of statistical differentials^{24,29} where the variance of proportion of parous flies was estimated using the normal approximation.

Seasonal transmission potential. Because no collections were undertaken outside of the peak transmission season, it was not possible to calculate the annual transmission potential (ATP). However, because the levels of transmission during the peak transmission season were very low (due to the effect of multiple rounds of ivermectin treatment), it is likely that transmission outside of the peak transmission season was zero or close to zero. Therefore, the seasonal transmission potential probably represents a good estimation of the ATP. The seasonal transmission potential was estimated by multiplying the MBR by the proportion of flies carrying infective stages of the parasite (i.e., parasites in the head capsule). This value was then multiplied by the estimated mean number of L3 larvae per infective fly. *Simulium ochraceum* s.l. typically has 1.5–2.0 *O. volvulus* L3 per infective fly in areas without intervention.³⁰ However, the number L3 per infective fly would be expected to decrease as the community microfilariae load decreases from ivermectin treatment,⁹ so this number after 17 rounds of treatment would be expected to be closer to 1. A value of 1 was therefore used in calculating monthly transmission potentials when analyzing the post-treatment data. This product was defined as the monthly transmission potential. Seasonal transmission potentials were then calculated by summing the monthly transmission poten-

tials. In all cases, estimates for which the 95% CIs did not overlap were considered to be significantly different at the $P < 0.05$ level.

RESULTS

The demographic characteristics of the seven sentinel communities are summarized in Table 1. Before commencing the ivermectin program, all sentinel communities from the endemic focus of southern Chiapas were classified as hyperendemic (prevalence of skin microfilariæ $\geq 60\%$). However, those in Oaxaca and northern Chiapas were either mesoendemic or hypoendemic.

A total of 54,933 *S. ochraceum* s.l. were collected at all sites, representing 312 collection days (Table 2). The overall proportion of parous females was 79% (range = 58–94%). Other species collected as part of this study (e.g., *S. callidum* and *S. metallicum* s.l.) were not analyzed further. Overall, 56% of the *S. ochraceum* s.l. pools (range = 23–100%) were tested by the PCR (Table 2). The proportion infected (i.e., positive bodies) and infective flies (i.e., positive heads) were then calculated from the proportion of positive pools and the number of flies per pool. The results of this analysis are summarized in Table 3. In general, the proportion of infective and infected flies from each site paralleled one another, although as expected, the proportion of infected flies was generally higher than the proportion of infective flies. The infection rate in Oaxaca was 22.0/10,000 (95% CI = 15–30), while the infective rate was 1.4/10,000 flies (95% CI = 0.26–4). Infected flies were not detected in northern Chiapas (95% CI = 0–5.3), but one pool of heads was found to be positive, leading to a calculated infective rate of 2.8/10,000 flies (95% CI = 0.08–14). In southern Chiapas infection rates were significantly higher than those in either northern Chiapas or Oaxaca (82.0/10,000; 95% CI = 57–114). The proportion of infective flies was also significantly higher than that found in Oaxaca and higher than that found in northern Chiapas (11.6/10,000; 95% CI = 4.2–25.3), although this difference was not statistically significant due to the wide 95% CI surrounding the infective prevalence estimate in northern Chiapas.

Mean intensity of infection (i.e., the estimated number of infective larvae per parous fly) was estimated from the PCR data as described in the Materials and Methods. The lowest

overall estimated mean intensity of infection was found in northern Chiapas (0.00048; 95% CI = 0.00046–0.00142 larvae per parous female). A low infection intensity was also found in Oaxaca (0.00395; 95% CI = 0.00283–0.00506). Intensities of infection in both northern Chiapas and Oaxaca were significantly lower ($P < 0.05$) than the intensity of infection in southern Chiapas (0.01269; 95% CI = 0.00871–0.01667).

Because flies were only collected during the peak transmission season, it was not possible to accurately estimate the ATP from the data. In place of the ATP, a seasonal transmission potential was calculated. However, because transmission outside of the peak season is likely to be extremely low or non-existent, it is likely that the seasonal transmission potential is a fairly accurate estimate of the ATP. Pre-treatment transmission data were available from one of our study communities (Morelos in southern Chiapas), based upon dissection of 6,819 flies.³¹ When these baseline data were compared with the seasonal transmission potential data derived from the pool screen PCR, transmission was estimated to have decreased by 97% (95% CI = 91–99%) as a result of treatment (Figure 1). The average number of L3s/fly in *S. ochraceum* s.l. is approximately two in areas without treatment and one in areas with treatment; therefore, a person living in the community during the 1981 and 2001 dry seasons received an estimated number of *O. volvulus* L3 larvae of 40 and 1, respectively. No pre-ivermectin data were available for the other foci. In Oaxaca, the overall seasonal transmission potential from the four communities was 0.53 (95% CI = 0.1–1.50) L3/person. In two communities, it was zero, and in another two, it was 0.66 and 2.0 L3/person (Table 3). The seasonal transmission potential in the community of Altigracia in northern Chiapas was 0.62 (95% CI = 0.1–3.1) L3/person, which may indicate evidence of autochthonous transmission, although this calculation was based upon a single PCR-positive head pool.

DISCUSSION

This is the first large-scale entomologic study using a PCR to measure transmission of *O. volvulus* in Mexico. The data presented above may be very useful to the Mexican National Onchocerciasis Control Program, which aims to eliminate onchocerciasis transmission with semi-annual treatments with ivermectin. This effort is based upon the hypothesis that ivermectin can reduce the skin microfilariæ community load to a level that results in a reduction of transmission to a rate that is below what is necessary for the parasite population to maintain itself. As mentioned in the introduction, this value (the level of transmission necessary to maintain parasite reproduction above the minimum reproductive rate) remains to be precisely defined. However, the transmission suppression effect of ivermectin is hypothesized to be particularly potent in areas where *S. ochraceum* s.l. is the vector, such as the foci in Mexico, because *S. ochraceum* s.l. is a facilitation-type vector that must feed on people with comparatively high microfilaridemia to develop significant numbers of infective-stage larvae.³² The data presented above demonstrate a dramatic reduction of transmission in the foci in Oaxaca and Chiapas, both of which have received multiple ivermectin treatments.

Given that mass delivery of ivermectin affects the microfilarial load in the skin of the human host population, and

TABLE 2

Simulium ochraceum s.l. collected and tested from the sentinel communities in Mexico

Focus/sentinel community	Catch days	Flies collected	Flies examined	% Parous
Oaxaca				
Santiago Teotlaxco	49	8,039	2,400	80
Santiago Lalopa	50	10,441	2,450	76
La Esperanza	30	15,036	10,650	58
La Chichina	48	6,450	6,450	77
Total	177	39,966	21,950	73
Southern Chiapas				
Morelos	50	5,894	3,450	77
Ampliación Malvinas	41	2,631	1,850	94
Total	91	8,525	5,300	86
Northern Chiapas				
Altigracia	44	6,442	3,650	77
Grand total	312	54,933	30,900	79

TABLE 3

Infection prevalence, infectious prevalence, and seasonal transmission potential in *Simulium ochraceum* s.l. at the sentinel communities in Mexico*

Focus/sentinel community	Infection rate (per 10,000 parous flies)	Infective rate (per 10,000 parous flies)	Estimated mean number of larvae per parous fly	Seasonal transmission potential
Oaxaca				
Santiago Teotlaxco	68.0 35–118	8.5 1.0–29.8	0.0056 0.0027–0.0084	2.00 0.20–7.00
Santiago Lalopa	67.0 34–115	0.0 0.0–7.8	0.0042 0.0020–0.0064	0.00 0–2.50
La Esperanza	17.6 9.8–28.6	0.9 0.03–4.8	0.00519 0.0029–0.0075	0.66 0.02–3.5
La Chichina	0.0 0–2.9	0.0 0–2.9	0.0 0.0–0.0	0.00 0.00–0.70
Total	22.0 15.0–30.0	1.4 0.26–4.0	0.0040 0.0028–0.0051	0.53 0.10–1.50
Southern Chiapas				
Morelos	81.0 48–125	5.9 0.6–21.2	0.0121 0.0072–0.0170	1.02 0.10–3.70
Ampliación Malvinas	86.0 43–151	22.8 5.8–58.1	0.0154 0.0079–0.0230	2.23 0.60–5.60
Total	82.0 57–114	11.6 4.2–25.3	0.0127 0.0087–0.0167	1.53 0.5–3.3
Northern Chiapas				
Altagracia	0.0 0–5.3	2.8 0.08–14	0.00048 0.0005–0.0014	0.62 0.1–3.1
Grand total	28.0 22–36	3.2 1.5–5.9	0.0048 0.0038–0.0058	0.78 0.4–1.4

* In each cell, the top number in **bold** represents the estimated value and the lower numbers represent the 95% confidence interval surrounding the estimated value.

therefore parasite uptake by the simuliid vector,^{33,34} it has been suggested that entomologic indicators of impact of ivermectin on transmission be based upon the prevalence of all forms of the parasite found in the vector, and not just infective larvae alone.^{20,35} In this way, changes in infection rates would more directly reflect levels of ivermectin coverage and compliance.⁵ The PCR analysis of fly bodies permits the detection of the developing larval states, providing estimates of the degree of contact between the vector and the infected human population. The infection rates in La Chichina, Oaxaca, and Altagracia in northern Chiapas were zero, indicating that ivermectin coverage has dramatically reduced the number of microfilariae available for transmission. In this study, the mean larval load per parous fly was also estimated by a mathematical model,²⁰ using as a starting point the rate of infection in parous fly bodies and heads as measured by the PCR. Given that in onchocerciasis parasite load correlates better with morbidity in host populations than do infection rates,³⁶ control programs might benefit if an estimation of the larval infection intensity in local vector population is available.

Infections were found in *S. ochraceum* s.l. collected in both Chiapas and Oaxaca. In both states, it is clear that transmission is not evenly distributed, since we found wide differences in the parous infection rate (body pools) and infective rate with (head pools) from different catch points in the same state. For example, in Morelos in southern Chiapas, the infective rate in the parous population was 5.9 per 10,000 parous flies, while the infective rate in Ampliación Malvinas was more than three times higher (22.8 per 10,000 parous flies). Similarly, the infective rate in Oaxaca varied from 0 to 8.5 per 10,000 parous flies. The persistence of transmission in Ampliación Malvinas was in concordance with previous studies,^{10,11} which have documented infections in children \leq 5

years old, as well as additional exposure of this population to the parasite, as judged by the presence of *O. volvulus* reactive antibodies in sera collected from these children.¹⁰

Overall, vector infection and infective rates were higher in southern Chiapas than in the other two foci. One factor that may be contributing to the relatively high level of ongoing transmission of *O. volvulus* in southern Chiapas in spite of the apparently successful ivermectin distribution effort is the presence of a significant migrant population at this focus. Migrant laborers working on the coffee plantations are not currently targeted for ivermectin treatment, yet this group may represent 40% of the total population of Southern Chiapas during the peak transmission season (Rodríguez-Pérez MA and others, unpublished data). Migrants may therefore represent a significant parasite reservoir that has yet to be efficiently targeted by the current ivermectin distribution program.^{2,37}

Because baseline data do not exist for Oaxaca, it is not possible to quantitate the effect of ivermectin on transmission in this focus. However, as mentioned in the Introduction, World Health Organization criteria for the interruption of transmission in areas in South and Central America for which baseline data are not available has been set at a threshold of less than 1 in 10,000 flies infected.⁴ In west Africa, this threshold had been set by the World Health Organization at 1 in 1,000 parous flies' based upon modeling studies carried out in areas in which *S. damnosum* s.l. is the vector.³⁸ In South America, the threshold has been set roughly at an order of magnitude lower than this value due to the higher biting rate of *S. ochraceum* s.l. From the data presented earlier in this report, it is possible that transmission may already be at or below this conservative threshold at all four communities in Oaxaca, the single community examined in northern Chiapas, and one of the two communities examined in southern Chia-

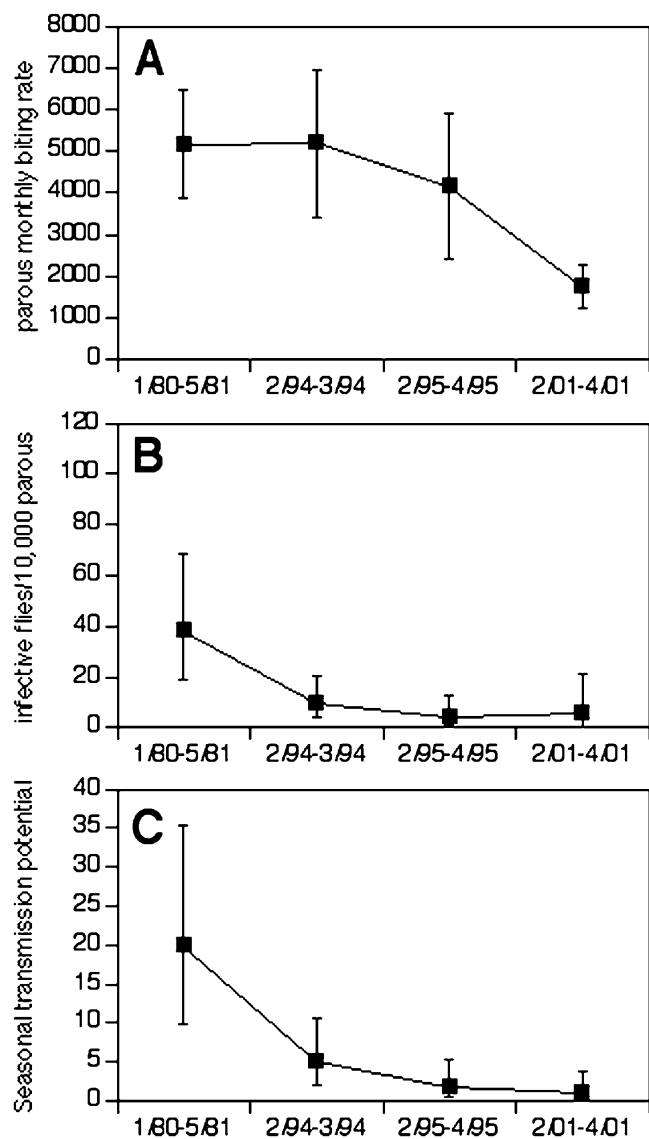


FIGURE 1. Effect of ivermectin treatment on transmission of *Onchocerca volvulus* in Morelds, Southern Chiapas, Mexico. **A**, Monthly parous biting rates. Points represent means and error bars represent standard deviations around the means of the parous biting rates at Morelds at four periods when surveys were conducted at this site. **B**, Infective rate (per 10,000 parous flies). Points represent estimates of the proportion of flies carrying infective larvae (expressed as number per 10,000 flies) and error bars represent the 95% confidence interval surrounding these estimates. **C**, Seasonal transmission potential. Points represent estimates of the seasonal transmission potential (calculated as described in the Materials and Methods) and error bars represent the 95% confidence intervals surrounding these estimates. In each panel, 1/81-5/81 represents pre-treatment values, 2/94-3/94 values after five rounds of ivermectin treatment, 2/95-4/95 values after six rounds of ivermectin treatment, and 2/01-4/01 values after 17 rounds of ivermectin treatment.

pas because the lower bound of the 95% CIs for the estimates of the infective rate in these communities are all at or below 1 in 10,000. However, the upper bounds for the 95% CIs of the estimated infective rate remain above this threshold in all seven communities. It will be necessary to examine additional flies, especially in the communities where no infective flies have been found, to more accurately determine if they fall below the 1/10,000 threshold. However, it should be noted

that the hypothesis that transmission may have been interrupted in Oaxaca is supported by parasitologic and serologic data reported from this area.^{3,16} Results of serologic tests and skin biopsies in children from Oaxaca were all negative, so transmission in Oaxaca appeared to be suppressed by these criteria.³⁹ One possible reason for the relative success of the program in Oaxaca when compared with southern Chiapas is that human migration in this area is lower. Thus, migrant populations that are currently not included in the distribution program may form a less significant human reservoir for the parasite than in southern Chiapas.

The finding of a single PCR-positive head pool in northern Chiapas suggests that autochthonous transmission may be occurring in this area. However, no infected body pools were found in the samples from northern Chiapas. This was surprising because the rate of PCR positivity in body pools is generally 4–20 times greater than the rate of PCR positivity in head pools (see Table 3 and Guevara and others⁷). It is therefore possible that transmission is not occurring in northern Chiapas, and that the finding of a single positive head pool from this area was a laboratory artifact. There are plans to test the remaining 43% of the flies collected from this focus to address this question.

In Ecuador, *O. volvulus* transmission has been completely interrupted in a former *O. volvulus*-endemic area, where *S. exiguum* is the vector.⁷ *Simulium exiguum* is a highly competent limitation-type vector. In contrast, the data presented earlier in this report suggest that parasite transmission in Mexico has not been interrupted, although in some communities it may have been suppressed. This is despite the fact that the Mexican program is using the same strategy of multiple semi-annual treatments with ivermectin found to be so successful in Ecuador, and despite the fact that in Mexico the vector is an armed species with a lower vector competence than *S. exiguum*. There are at least two possible reasons for this. First, as mentioned earlier, a significant migrant laborer population exists that has yet to be effectively targeted by the current ivermectin distribution program. Second, ivermectin distribution has not been equally efficient in all affected communities. Some of the endemic communities within each focus have not received regular semi-annual treatment, while in others that have had regular semi-annual treatments less than 85% of the eligible population have received treatment. One reason for this relatively low depth of coverage is that community microfilariae loads are extremely low in some communities. In these communities the disease burden is also quite low, so people generally do not feel sick, and are consequently less interested in complying with the distribution program. It is possible that such non-compliant individuals may be carrying significant levels of skin microfilaria and may continue to support transmission even in the face of a high overall rate of coverage in the rest of the community. In such cases, enhanced efforts may be needed to identify such individuals and to convince them to be treated, to succeed in interrupting transmission.

In summary, the data presented demonstrate that the Mexican onchocerciasis control program has resulted in a dramatic reduction in the level of transmission of *O. volvulus* at all three foci in the country. However, transmission still appears to be continuing in some communities, particularly in areas with a high migrant population. To reach the goal of interrupting transmission completely, it may therefore be neces-

sary for the program to adjust its strategic approach slightly, by giving more attention to the treatment of migrants and non-compliant individuals.

Received August 11, 2003. Accepted for publication September 26, 2003.

Acknowledgments: We thank Marco Sandoval, Rafael Vázquez and Juan Ventura of Instituto Nacional de Salud Pública at Centro de Investigación de Paludismo for assisting in the field work. We are in debt to Dr. Olga Real Najarro (Universidad Valle del Bravo) for comments and constructive criticisms on an earlier version of the manuscript.

Financial support: This project was supported by the Consejo Nacional de Ciencia y Tecnología (grant 34486-M: molecular methods to determine the incidence levels of *Onchocerca volvulus* and the parasite transmission in Mexico), and by the Onchocerciasis Elimination Program for the Americas (OEPA).

Authors' addresses: Mario A. Rodríguez-Pérez, Cristian Lizarazo-Ortega, and Alberto Mendoza-Herrera, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Boulevard del Maestro Esquina Elías Piña, Col. Narciso Mendoza, 88710, Reynosa, Tamaulipas, Mexico. Bruce G. Lilley and Thomas R. Unnasch, Division of Geographic Medicine, BBRB Box 7, University of Alabama at Birmingham, 1530 3rd Avenue South, Birmingham, AL 35294, E-mail: trunnasch@geomed.dom.uab.edu. Alfredo Domínguez-Vázquez, Dirección de Servicios de Salud, Instituto de Salud del estado de Chiapas, Unidad Administrativa, Edificio C. de la Calzada a la Unidad Deportiva, 29007, Tuxtla Gutiérrez, Chiapas, Mexico. Ramón Segura-Arenas, Programa de Oncocercosis Oaxaca, Dirección de Servicios de Salud, Secretaría de Salud de Oaxaca, JP García 807, Centro, 68000, Oaxaca, Oaxaca, Mexico. Filiberto Reyes-Villanueva, Laboratorio de Entomología Médica, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, 66450, San Nicolás de los Garza, NL Mexico.

Reprint requests: Mario A. Rodríguez-Pérez, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Boulevard del Maestro Esquina Elías Piña, Col. Narciso Mendoza, 88710, Reynosa, Tamaulipas, México, Telephone/Fax : 52-899-924-3627, E-mail: mar@mail.cbipn.mx.

REFERENCES

- World Health Organization, 1991. *Executive Summary*. First Inter American Conference on Onchocerciasis. Washington, DC: Pan American Health Organization/World Health Organization.
- World Health Organization, 1995. Onchocerciasis and its control. *World Health Organ Tech Rep Ser* 852.
- IACO, 2001. *Relatoría de la XI Conferencia Interamericana sobre Oncocercosis (IACO)*. Mexico, D. F. Noviembre 27–29, 2001.
- World Health Organization, 2001. *Certification of Elimination of Human Onchocerciasis: Criteria and Procedures*. Geneva: World Health Organization. Document WHO/CDS/CPE/CEE/2001.
- Cupp EW, Ochoa JO, Collins RC, Cupp MS, González-Peralta C, Castro J, Zea-Flores G, 1992. The effects of repetitive community-wide ivermectin treatment on transmission of *Onchocerca volvulus* in Guatemala. *Am J Trop Med Hyg* 47: 170–180.
- Guderian RH, Anselmi M, Espinel M, Mancero T, Rivadeneira G, Proaño R, Calvopiña HM, Vieira JC, Cooper PJ, 1997. Successful control of onchocerciasis with community-based ivermectin distribution in the Rio Santiago focus in Ecuador. *Trop Med Int Health* 2: 982–988.
- Guevara AG, Viera JC, Lilley BG, López A, Vieira N, Rumbia J, Collins R, Katholi CR, Unnasch TR, 2003. Entomological evaluation by pool screen polymerase chain reaction of *Onchocerca volvulus* transmission in Ecuador following mass Mectizan distribution. *Am J Trop Med Hyg* 68: 222–227.
- Hougar JM, Alley ES, Yamèogo L, Dadzie KY, Boatman BA, 2001. Eliminating onchocerciasis after 14 years of vector control: a proved strategy. *J Infect Dis* 184: 497–503.
- Rodríguez-Pérez MA, Rodríguez MH, Margeli-López HM, Rivás-Alcalá AR, 1995. Effect of semiannual treatments of ivermectin on the prevalence and intensity of *Onchocerca volvulus* skin infection, ocular lesions, and infectivity of *Simulium ochraceum* populations in southern Mexico. *Am J Trop Med Hyg* 52: 429–434.
- Rodríguez-Pérez MA, Danis-Lozano R, Rodríguez MH, Bradley JE, 1999a. Comparison of serological and parasitological assessments of *Onchocerca volvulus* transmission after 7 years of mass ivermectin treatment in Mexico. *Trop Med Int Health* 4: 98–104.
- Rodríguez-Pérez MA, Danis-Lozano R, Rodríguez MH, Unnasch TR, Bradley JE, 1999b. Detection of *Onchocerca volvulus* infection in *Simulium ochraceum sensu lato*: Comparison of a PCR assay and fly dissection in a Mexican hypoendemic community. *Parasitology* 119: 613–619.
- Brandling-Bennett AD, Anderson J, Fuglsang H, Collins RC, 1981. Onchocerciasis in Guatemala. Epidemiology in fincas with various intensities of infection. *Am J Trop Med Hyg* 30: 970–981.
- Rodríguez-Pérez MA, Reyes-Villanueva F, 1994. Efecto de la ivermectina sobre la transmisión de *Onchocerca volvulus* en México. *Salud Publica Mex* 36: 281–290.
- Vázquez-Castellanos JL, 1991. Cafecultura e historia social de la oncocercosis en el Soconusco, estado de Chiapas, México. *Salud Publica Mex* 33: 124–135.
- Davies JB, 1968. A Review of Past and Present Aspects of *Simulium Control in Mexico Together with Recommendations for the Future Conduct of Control Schemes and an Outline of an Eradication Scheme in the North Focus of Onchocerciasis in Chiapas State*. Washington, DC: Pan American Health Organization.
- Martín-Tellaache A, Ramírez-Hernández J, Santos-Preciado JI, Galván J, 1998. Onchocerciasis: changes in transmission in Mexico. *Ann Trop Med Parasitol* 1: S117–S119.
- Meredith SEO, Lando G, Gbakima A, Zimmerman PA, Unnasch TR, 1991. *Onchocerca volvulus*: application of the polymerase chain reaction to identification and strain differentiation of the parasite. *Exp Parasitol* 73: 335–344.
- Zimmerman PA, Dadzie KY, DeSole G, Remme J, Soumbeiy-Alley E, Unnasch TR, 1992. *Onchocerca volvulus* DNA probe classification correlates with epidemiological patterns of blindness. *J Infect Dis* 165: 964–968.
- Zimmerman PA, Toè L, Unnasch TR, 1993. Design of *Onchocerca* DNA probes based upon analysis of a repeated sequence family. *Mol Biochem Parasitol* 58: 259–268.
- Basáñez MG, Rodríguez-Pérez MA, Reyes-Villanueva F, Collins RC, Rodríguez MH, 1998. Determination of sample sizes for the estimation of *Onchocerca volvulus* (Filarioidea: Onchocercidae) infection rates in biting populations of *Simulium ochraceum* s. l. (Diptera: Simuliidae) and its application to ivermectin control programs. *J Med Entomol* 35: 745–757.
- Katholi CR, Toé L, Merriweather A, Unnasch TR, 1995. Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies. *J Infect Dis* 172: 1414–1417.
- Rodríguez-Pérez MA, Rivás-Alcalá R, 1992. Age structure of *Simulium ochraceum* and transmission of *Onchocerca volvulus* in Mexico. Clark GG, Suárez MF, organizers. *Mosquito Vector Control and Biology in Latin America – A Second Symposium*. *J Am Mosq Control Assoc* 8: 305–317.
- Cupp EW, Collins RC, 1979. The gonotrophic cycle in *Simulium ochraceum*. *Am J Trop Med Hyg* 28: 422–426.
- Grillet ME, Basáñez MG, Vivas-Martínez S, Villamizar N, Frontado H, Cortez J, Coronel P, Botto C, 2001. Human onchocerciasis in the Amazonian area of southern Venezuela: spatial and temporal variation in biting and parity rates of black flies (Diptera: Simuliidae) vectors. *J Med Entomol* 38: 520–530.
- Unnasch TR, Meredith SEO, 1996. The use of degenerate primers in conjunction with strain and species oligonucleotides to classify *Onchocerca volvulus*. Clapp JP, ed. *Methods in Molecular Biology*. Volume 50. *Species Diagnostics Protocols*:

- PCR and Other Nucleic Acid Methods*. Totowa, NJ: Humana Press, Inc., 293–303.
26. Nutman TB, Zimmerman PA, Kubofcik J, Kostyu DD, 1994. A universally applicable diagnostic approach to filarial and other infections. *Parasitol Today* 10: 239–243.
 27. Yamèogo L, Toè L, Hougard JM, Boatín BA, Unnasch TR, 1999. 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. *Am J Trop Med Hyg* 60: 124–128.
 28. Walsh JF, Davies JB, LeBerre R, Garms R, 1978. Standardization of criteria for assessing the effects of *Simulium* control in onchocerciasis control program. *Trans R Soc Trop Med Hyg* 72: 675–676.
 29. Kotz S, Johnson NL, Read CB, 1988. Method of statistical differentials. *Encyclopedia of Statistical Sciences*. New York: John Wiley & Sons, 646–647.
 30. Cupp EW, 2003. Vector species, transmission efficiency, and frequency of ivermectin treatment. Dadzie Y, Neira M, Hopkins D, eds. *Final Report of the Conference on Eradicability of Onchocerciasis*. 51–52.
 31. Ortega M, Oliver M, Ramírez A, 1992. Entomología de la onchocercosis en el Soconusco, Chiapas. VI. Estudios cuantitativos de la transmisión de *Onchocerca volvulus* por tres especies de simúlidos en una comunidad de alta endemia. *Rev Fac Med UNAM* 35: 95–103.
 32. Collins RC, Lehmann T, Vieira-García JC, Guderian RH, 1995. Vector competence of *Simulium exiguum* for *Onchocerca volvulus*: implications for the epidemiology of onchocerciasis. *Am J Trop Med Hyg* 52: 213–218.
 33. Cupp EW, Ochoa JO, Collins RC, Ramberg FR, Zea-Flores G, 1989. The effect of multiple ivermectin treatments on infection of *Simulium ochraceum* with *Onchocerca volvulus*. *Am J Trop Med Hyg* 40: 501–506.
 34. Basañez MG, Boussinesq M, Pröheon J, Frontado H, Villamizar NJ, Medley GF, Anderson RM, 1994. Density-dependent processes in the transmission of human onchocerciasis: intensity of microfilariae in the skin and their uptake by the simuliid host. *Parasitology* 108: 115–127.
 35. Basañez MG, Remme JHF, Alley ES, Bain O, Shelley AJ, Medley FG, Anderson RM, 1995. Density-dependent processes in the transmission of human onchocerciasis: relationship between the numbers of microfilariae ingested and successful larval development in the simuliid vector. *Parasitology* 110: 409–427.
 36. Anderson RM, May RM, 1985. Helminth infections of humans; mathematical models, population dynamics, and control. *Adv Parasitol* 24: 1–101.
 37. Boatín B, Molyneux DH, Hougard JM, Christensen OW, Alley ES, Yamèogo L, Seketeli A, Dadzie KY, 1997. Patterns of epidemiology and control of onchocerciasis in West Africa. *J Helminthol* 71: 91–101.
 38. Remme JHF, 1995. Estimation and prediction in tropical disease control: the example of onchocerciasis. Mollison D, ed. *Epidemic Models: Their Structure and Relation to Data*. Cambridge, United Kingdom: Cambridge University Press, 372–392.
 39. Meredith SEO, Dull HB, 1998. Onchocerciasis: the first decade of mectizan™ treatment. *Parasitol Today* 14: 472–474.