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Real-Time Polymerase Chain Reaction Method for the Detection of *Onchocerca volvulus* in Post-Elimination Surveillance of Onchocerciasis in Ecuador

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Abstract. Onchocerciasis has been declared eliminated in Ecuador and surveillance measures are of great interest. In this study, we examined the infectivity rates of *Simulium exiguum* by *Onchocerca volvulus* in previously hyperendemic areas in Esmeraldas province of Ecuador. These areas had previously undergone mass administration of ivermectin, which led to the interruption of transmission in 2009 and the certification of elimination in 2014. The study included three communities in Río Cayapas and one in Río Canandé, and a total of 2,950 adult *S. exiguum* were collected in 2018. We used quantitative polymerase chain reaction with *O. volvulus* O-150 plasmid control DNA to analyze 59 pools. Our findings revealed that the infectivity rates were zero, indicating that the transmission of *O. volvulus* remained suspended in the area.

Onchocerciasis is a disease caused by the filarial parasite worm *Onchocerca volvulus* known as "river blindness." It is characterized by the presence of subcutaneous nodules harboring adult parasites and the presentation of a dermatitis that can be extremely severe visual impairment, and in some cases blindness, distributed in foci in the Americas and sub-Saharan Africa.[1,2] In Latin America, a strategy of periodic mass drug administration (MDA) with ivermectin in the community has led to certification of elimination in endemic foci in Colombia, Ecuador, Mexico, and Guatemala, although transmission is still active in an isolated rainforest focus on the Brazil–Venezuela border.[3] In Ecuador, ivermectin was administered annually or biannually to communities in endemic foci in the province of Esmeraldas from 1991 to 2009, when treatments were discontinued after interruption of transmission.[4,5] Posttreatment surveillance of black flies by polymerase chain reaction (PCR) for *O. volvulus* DNA (using O-150 PCR) in 2012 showed no evidence of active transmission.[6] Onchocerciasis was certified eliminated in Ecuador in 2014. Measurement of infectivity rates in vectors by O-150 PCR is considered the key indicator for detection of interruption and resurgence of transmission.[7] Assessment of exposure to *O. volvulus* by detecting antibodies to *O. volvulus*–specific antigen, Ov16, is also useful.[8,9] This study was designed to determine the entomological indices and infectivity rate of onchocerciasis transmission after 9 years of massive treatment with ivermectin in three communities of the Cayapas river and one community of the Canandé river.

All selected enclaves have hydrological profiles as ex-endemic rainforest areas for onchocerciasis in the province of Esmeraldas, Ecuador.[3] Collection of simulid species was conducted in four communities considered to be previously hyperendemic for onchocerciasis, including San Miguel (0°47′47.2′′N 78°55′04.0′′W), El Tigre (0°44′17.2′′N 78°56′40.2′′W), Corriente Grande (0°41′02.0′′N 78°57′29.3′′W) and El Naranjal (0°26′43.8′′N 79°18′00.7′′W) (Figure 1). Sampling was conducted during July 2018, following the guidelines for entomological evaluation for certification of human onchocerciasis elimination.[10,11] The collection of simulids was performed by two people by exposing a leg section and collected any black fly landing on leg before it took a blood meal using a mouth aspirator. Collections were carried out from 7:00 AM to 18:00 PM at 1-hour intervals, labeling the day, month, community, and number of flies collected.[12] All specimens collected were stored in 2-mL vials containing 95% ethanol and transported to the Centro de Referencia Nacional de Vectores, of the Instituto Nacional de Investigación en Salud Pública (INSPI), for morphological identification according to the taxonomic key of Coscarón and Coscarón.[13] Each specimen was classified by morphotype and by feeding status (fed or not). For molecular analyses, only individuals identified as *Simulium exiguum* were selected because this species is the main vector of *O. volvulus*. *Simulium quadrivittatum*, a minor vector also present in the area was not included in the study. *Simulium exiguum* pools of 50 individuals were formed in 1.5-mL vials in 95% alcohol. The heads were then separated from the bodies of every insect vector manually with the aid of a stereo microscope, entomological forceps, and dissecting needles. They were also preserved in 95% ethanol. The number of individuals was calculated to be able to detect the possibility of an infectivity rate of one in 2000 uninfected individuals.[6] DNA extraction was performed with the Wizard SV Genomic DNA Purification System Kit at the University of Salamanca and the NucleoSpin Tissue, Macherey–Nagel kit at INSPI and the analysis was based on PCR O-150. All PCRs were carried out in sets of 59 samples, in rows B–H of a PCR microtiter plate. A volume of 2.5 μL of the purified genomic DNA was used as a template for the PCR amplifications carried out in a total volume of 50 μL containing 0.5 μM of O-150 primer (5′-GATTYTTCCGRCGAANARC GC-3′) and 0.5 μM of biotinylated O-150 primer (5′-B-GCNRTRTAAATNTGNAAATTC-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 $(NH_4)_2SO_4$, 2 mM $MgCl_2$, and 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 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 6 minutes.[7] An *O. volvulus* O-150 plasmid was used as a positive control. All samples were analyzed in duplicate. The frequency of infective and infected black flies, taken from clusters of black fly heads and bodies, respectively, was determined using the Pool screen algorithm.

Our findings revealed that the frequency of infectious black flies was 0 out of 19 pools in the community El Tigre, 0 out of 14 pools in the community San Miguel, 0 out of 11 pools in the community Naranjal, and 0 out of 15 pools in the community Corriente Grande (Table 1). DNA was extracted from 36 pools in Ecuador and from 23 pools in Spain. No *O. volvulus* signal was found in any of the 59 pools analyzed, so the infectivity rate was 0% (Figure 2).

Post-elimination surveillance is carried out following the Sistema Nacional de Vigilancia Epidemiológica (SIVE-Alerta) of the Ministerio de Salud Pública to detect a possible recrudescence. Since its certification, from 2014 to 2018, no cases of onchocerciasis in Ecuador have been reported. In 2018, a serum evaluation study with ELISA test was performed on 123 children aged 5 to 9 years living in four communities (Zapallo Grande, San Miguel, El Tigre, and Corriente Grande) previously considered to be hyperendemic for onchocerciasis in the province of Esmeraldas in Ecuador. All samples were negative, indicating no evidence of transmission of infection.[8] Molecular assays (quantitative PCR [qPCR]) can be used to monitor the resurgence of *O. volvulus* transmission. It would be expected that there may be the possibility of finding infected flies due to the constant population migration to the Americas region (Brazil and Venezuela) and Africa, areas that are still endemic for onchocerciasis.[14] This study, conducted in communities previously considered hyperendemic for onchocerciasis in Ecuador, which were the last to receive ivermectin MDA in the country, confirms the absence of *O. volvulus* transmission in the communities under study.

Current guidelines for the use of qPCR testing are to confirm interruption of transmission as part of post-treatment surveillance when the results of fly infectivity rates show levels close to the threshold for interruption of transmission. Recommendations for post-elimination surveillance include periodic testing of fly infectivity by O-150 PCR until

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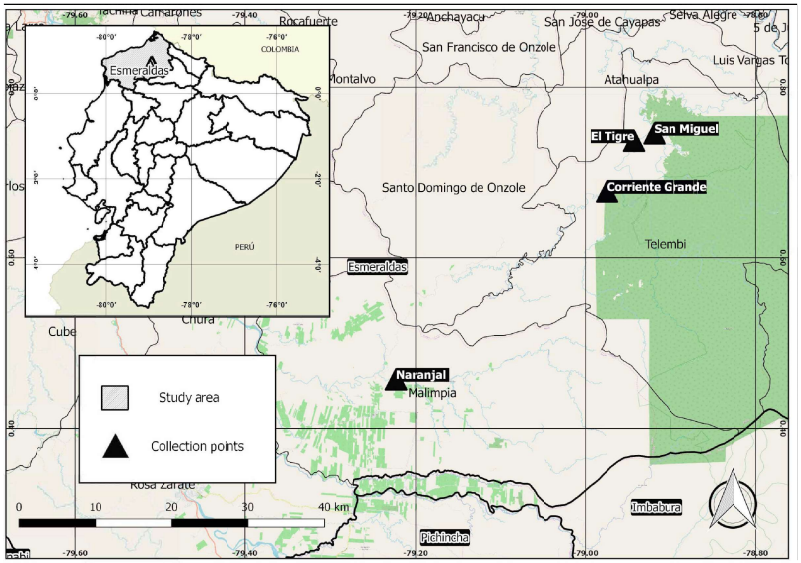

FIGURE 1. Location study communities and *Simulium exiguum* collection sites in former onchocerciasis hyperendemic communities in Esmeraldas's province of Ecuador. This figure appears in color at www.ajtmh.org.

TABLE 1
Pool and specimen collection with percentages of *Simulium exiguum* adults in onchocerciasis ex-endemic communities

Ex-endemic communities	Total pools formed	No. of specimens	Percentage
Río Cayapas			
Corriente Grande	15	750	25.4
San Miguel	14	700	23.7
El Tigre	19	950	32.2
Río Canandé			
El Naranjal	11	550	18.6
Total	59	2,950	100.0

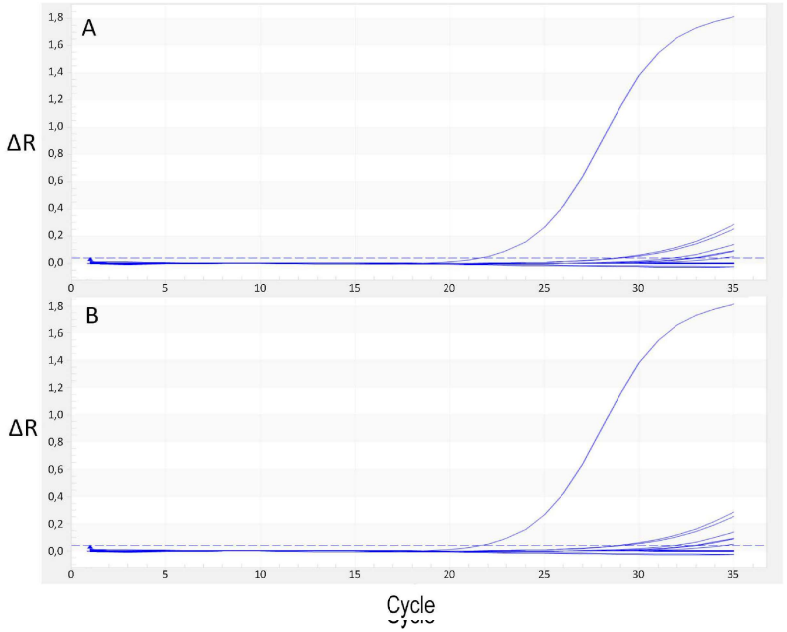

FIGURE 2. *Simulium exiguum* pool samples tested by quantitative polymerase chain reaction using the O-150 sequence showing fluorescence signal over time. (**A**) Results of the 36 pool samples processed at Instituto Nacional de Investigación en Salud Pública. (**B**) Results of the 23 pool samples processed at the University of Salamanca. All pool samples analyzed were negative. The only amplification curve detected in both analyses corresponds to the amplification of the sample used as positive control *Onchocerca voluvuls* O-150 sequence in a plasmid. This figure appears in color at www.ajtmh.org.

elimination is verified in all endemic countries in the same region.[3] Unfortunately, the unavailability of DNA obtained from native *O. volvulus* is a limitation of our study, which was addressed using the O-150 sequence in a plasmid, which must be taken into account when interpreting the data. However, conducting black fly collections and O-150 PCR after certification of elimination is a challenge for most formerly endemic countries, including Ecuador, with multiple competing demands on scarce health resources: maintaining such capacity requires continued investment in infrastructure and personnel. In the case of the latter, effective vector surveillance requires experience and expertise, as well as historical knowledge of where and when to sample vectors for transmission.[10] In the specific case of Ecuador, after elimination certification, the entomological expertise of the program was lost due to the reassignment or loss of experienced personnel and the lack of an allocated budget for post-certification activities. Therefore, it is important to maintain Ov16 ELISA-based surveillance in humans, accompanied by entomological evaluation (qPCR) regularized in a surveillance protocol to monitor the recrudescence of transmission in onchocerciasis formerly endemic areas.[2,6]

In conclusion, we have used qPCR to monitor the possible resurgence of *O. volvulus* transmission in previously hyperendemic communities after 9 years of onchocerciasis elimination certification in Ecuador. Results obtained from black flies show no evidence of *O. volvulus* transmission from 2009 to 2018 when MDA was stopped. We believe that qPCR-based surveillance is important to continue the practice on vector infectivity measurements for post-certification surveillance accompanied by Ov16 serological testing.

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