Molecular Identification of *Onchocerca* spp. Larvae in *Simulium damnosum* sensu lato Collected in Northern Uganda

Guilherme G. Verocai,^{1,2} Hassan K. Hassan,¹ Thomson Lakwo,³ Peace Habomugisha,⁴ Moses N. Katabarwa,⁵ Stephen Begumisa,⁶ Philbert Clouds,⁷ James Katamanywa,⁸ Christine Nahabwe,³ and Thomas R. Unnasch^{1*}

¹Global Health Infectious Diseases Research Program, Department of Global Health, College of Public Health, University of South Florida, Tampa, Florida; ²Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia; ³Vector Control Division, Ministry of Health, Kampala, Uganda; ⁴The Carter Center, Uganda Office, Kampala, Uganda; ⁵The Carter Center, Atlanta, Georgia; ⁶Rubirizi District Health Services, Rubirizi, Uganda; ⁷Kabarole District Health Services, Kabarole District, Uganda; ⁸Kyenjojo District Health Services, Kyenjojo District, Uganda

Abstract. Previous studies have demonstrated that the presence of larvae of other filarial species in *Simulium damnosum* sensu lato can distort estimates of transmission potential for *Onchocerca volvulus* in West Africa. However, studies conducted in foci of onchocerciasis in West Central Uganda indicated that larvae other than *O. volvulus* were not common in vectors collected there. Recent data collected in Northern Uganda revealed a striking discordance between estimates of the prevalence of flies carrying *O. volvulus* infective larvae obtained from molecular pool screening and dissection methods. To resolve this discrepancy, sequences from three mitochondrially encoded genes were analyzed from the larvae collected by dissection. All larvae analyzed were *Onchocerca ochengi* v. Siisa, a parasite of cattle, or *Onchocerca ramachandrini*, a parasite of warthogs. These results suggest that nonhuman parasite larvae are common in vectors in Northern Uganda, underscoring the necessity for molecular identification methods to accurately estimate *O. volvulus* transmission.

In 2007, Uganda announced a program to eliminate onchocerciasis in all 17 foci in the country,¹ becoming the first country in Africa to make complete elimination of onchocerciasis a national goal. The strategic plan of the Uganda Onchocerciasis Elimination Program was based on twice per year mass drug administration of Mectizan (ivermectin) to the afflicted communities supplemented with localized vector control measures where appropriate. In 2008, the Uganda Ministry of Health established an advisory committee, known as the Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEEAC) to provide technical guidance to the elimination program. One of the first actions of the UOEEAC was to consult with the Ugandan Ministry of Health to develop a set of guidelines for the program to use for verifying the suppression and eventual elimination of transmission of *Onchocerca volvulus*.² These guidelines were based on the guidelines for the elimination of onchocerciasis published by the World Health Organization in 2001³ and updated in 2016.⁴ The guidelines rely on a combination of serological indicators of transmission (development of antibodies against the parasite specific antigen Ov16 in children) and entomological indicators (presence of infective stage larvae in the black fly vectors of the parasite) to confirm suppression of transmission.

Detection of infective larvae in the vector black flies has advantages when used to monitor transmission of *O. volvulus*. Most importantly, it provides the most timely and accurate measure of transmission. Traditionally, detection of infective larvae in vectors has been accomplished through dissection of vector black flies. However, there are some disadvantages associated with the method. First, in the face of an effective control or elimination program, flies carrying infective larvae become increasingly rare. This means that large numbers of flies need to be examined to detect the rare infectious fly, which is laborious and expensive. Second, in West Africa, it

has long been known that *Simulium damnosum* s.l., the most important vector throughout Africa also serves as the vector for a number of *Onchocerca* species associated with domestic or wild ungulate hosts (e.g., bovids, suids). These larvae are difficult or impossible to distinguish from *O. volvulus* morphologically, confounding accurate measurement of the prevalence of flies carrying infective larvae of the human parasite. Molecular methods involving polymerase chain reaction (PCR) amplification of *O. volvulus* specific DNA sequences in DNA prepared from pools of flies or from individual larvae have been developed to overcome these difficulties.^{5,6} When molecular identifications were performed on infective larvae identified by dissection in West Africa, it was found that about half of the larvae provisionally identified as *O. volvulus* by field dissection teams were ungulate-associated *Onchocerca*.⁵ By contrast, one report applying molecular identification methods to larvae collected from Kabarole and Kasese districts in West Central Uganda suggested that all were *O. volvulus*, suggesting that ungulate-associated *Onchocerca* might pose less of a problem in East Africa.⁷

Onchocerciasis elimination efforts in Northern Uganda were hindered by political unrest in the area for several years. However, after the restoration of peace to this area, elimination efforts accelerated in 2012. These efforts included both serosurveys of children to measure exposure to the parasite, as well as entomological surveys, in which a portion of the flies collected were screened in pools using molecular methods, whereas a portion were screened using traditional dissection. In 2015, significantly larger numbers of flies carrying infective larvae were detected by the field dissection teams than were found by PCR pool screening. To further investigate the reason for this discrepancy, the larvae collected by the dissection teams were subjected to a detailed molecular analysis.

Simulium damnosum females were collected using standard methods⁸ from several sites in the Madi-Mid North onchocerciasis focus of Northern Uganda (Figure 1). Flies were collected from the districts of Nwoya, Amuru (Elegu), and Moyo, as part of routine surveillance activities conducted by

* Address correspondence to Thomas R. Unnasch, Global Health Infectious Disease Research, 3720 Spectrum Boulevard Suite 304, Tampa, FL 33612. E-mail: tunnasch@health.usf.edu

FIGURE 1. Species identification of *Onchocerca* larvae isolates from black flies of Northern Uganda. (A) Maximum parsimony tree of mitochondrial 12S gene. (B) Maximum parsimony tree of mitochondrial ND5 gene (1,000 bootstrap replicates; bootstrap support values over 60% are shown besides tree branches). (C) Maximum parsimony tree of mitochondrial cytochrome c oxidase subunit 1 (COI) gene. In each panel results are shown for 2,000 bootstrap replicates; bootstrap support values over 60% are shown besides the tree branches.

the Uganda Onchocerciasis Elimination Program of the Uganda Ministry of Health. A total of 468 *S. damnosum* s.l. were collected over the course of the study. Of these, 286 were tested for the presence of *O. volvulus* L3 by pool screen PCR, whereas 182 were dissected by the field teams. The prevalence of flies carrying L3 as estimated by pool screen PCR was substantially lower than the number of flies found to be carrying L3 larvae by dissection (Table 1). This suggested that, unlike what was reported for foci in the west central part of Uganda, *S. damnosum* s.l. from the Madi-Mid North focus were likely to be carrying L3 that were misidentified as *O. volvulus* by the field teams. To test this hypothesis, 14 of the individual larvae detected by dissection were preserved and subjected to molecular species identification, using three different mitochondrially encoded genes. To accomplish this, genomic DNA was purified from the alcohol preserved larvae using the Qiagen DNeasy kit as per the manufacturer's instructions. PCR amplification of three mitochondrial gene fragments was performed following previously described protocols: 12S rRNA gene (forward primer -12SOvC: TCGGCTATGCGTT-TAATTTT; reverse primer-12SOvB: CAACTTACGCCCT-TAGGC), NADH dehydrogenase subunit 5 (ND5) (forward primer - ND5OvA: TTGGTTGCCTAAGGCTATGG; reverse primer - ND5OvC: CCCCTAGTAACAAACCAACCACA),⁹ and the cytochrome c oxidase subunit 1 (COI) (forward primer—COIF: TGATTGGTGGTTTTGGTAA; reverse primer—COIR: ATAAG-TACGAGTATCAATATC).¹⁰ PCR products for each of the three gene fragments were purified and sequenced using their respective primers using BigDye Terminator Cycle Sequencing (Applied Biosystems). Forward and reverse sequences were aligned and edited manually using FinchTV and MEGA version 7.¹¹ Phylogenetic analyses were performed separately

for each mitochondrial gene fragment (12S: 399–403 bp; ND5: 426–434 bp; COI: 591 bp) using the Maximum Parsimony (2,000 bootstrap replicates) method with the aim of identifying the *Onchocerca* species of each isolate (MEGA 7). Sequences of selected *Onchocerca* species available in GenBank were included in the analyses for comparison, and the canine onchocercids *Dirofilaria immitis* and *Dirofilaria repens*, as outgroups.

We obtained a total of 14, 11, and 13 sequences of the 12S, ND5, and COI regions, respectively. The results of the phylogenetic analysis of these sequences are presented in Figure 1. No *O. volvulus* larvae were found in the *S. damnosum* females. *Onchocerca* larvae were identified as *O. ochengi* “Siisa” form ($N = 13$) and a single *Onchocerca ramachandrinii* ($N = 1$, based on 12S and ND5 only), parasites of cattle and warthog, respectively (Figure 1, Panels A–C). *Onchocerca ochengi* is a common parasite of cattle throughout sub-Saharan Africa, and it is phylogenetically closely related to *O. volvulus*.¹² *Onchocerca ramachandrinii* is also a native African species infecting the common warthog (*Phacochoerus africanus*), a wild suid with a wide geographic range across sub-Saharan Africa.¹³ These results suggest that infection of *S. damnosum* s.l. with animal *Onchocerca* spp. is quite common in Northern Uganda, unlike what was previously reported in the west-central foci of the country. This difference may be attributable to differences in the ecology and vector populations between the northern and west-central foci in the country. For example, the vector present in the northern focus is the savanna dwelling sibling species of *S. damnosum* s.l., *Simulium sirbanum*, whereas both *Simulium neavei* and *S. damnosum* s.l. are present in the west-central foci.⁷ The different black fly vector species are known to exhibit different degrees of anthropophilicity.¹⁴ Regardless, these data support the findings of molecular identifications carried out over 20 years ago by the Onchocerciasis Control Program in West Africa⁵ and more recent studies performed in Northern Cameroon,¹⁵ which also demonstrated that a large proportion of larvae identified as *O. volvulus* by field dissection were misidentified, resulting in a dramatic overestimation of the intensity of transmission. These data highlight the necessity of using

TABLE 1

Comparison of prevalence of flies carrying L3 as estimated by pool screen PCR and dissection

Method	No. examined	No. positive	% positive	95% CI
Dissection	182	17	9.3	5.5–13.9%
Pool screen PCR	286 (6 pools)	0	0	0–0.6%

CI = confidence interval; PCR = polymerase chain reaction.

molecular methods capable of unambiguously identifying *O. volvulus* larvae, especially in areas where effective control measures are active and the prevalence of *O. volvulus* in the vector population is likely to be very low.

Received June 30, 2017. Accepted for publication August 17, 2017.

Published online October 2, 2017.

Financial support: This research was supported by funds provided by The Carter Center (project #6408109600).

Authors' addresses: Guilherme G. Verocai, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, E-mail: gverocai@gmail.com. Hassan K. Hassan and Thomas R. Unnasch, Global Health Infectious Disease Program, Tampa, FL, E-mails: hhassan@health.usf.edu and tunnasch@health.usf.edu. Thomson Lakwo and Christine Nahabwe, Vector Control Division, Ministry of Health, Kampala, Uganda, E-mails: tlakwo@gmail.com and ch.nahabwe@gmail.com. Peace Habomugisha, The Carter Center, Uganda office, Kampala, Uganda, E-mail: peace.habomugisha@cartercenter.org. Moses N. Katabarwa, The Carter Center, Atlanta, GA, E-mail: moses.katabarwa@cartercenter.org. Stephen Begumisa, Rubirizi District Health Services, Rubiriza, Uganda, E-mail: bgmsstephen@yahoo.com. Philbert Clouds, Vector Control Division, Kabarole, Uganda, E-mail: clouds.philbert@yahoo.com. James Katamanywa, Kyenjojo District Health Services, Kyenjojo, Uganda, E-mail: katamanywawilly@gmail.com.

REFERENCES

1. The Carter Center, 2007. *Uganda Launches Bold Policy to Eliminate River Blindness*. Eye of the Eagle, The Carter Center. https://www.cartercenter.org/resources/pdfs/news/health_publications/eye_of_eagle/uganda-launches-rb-policy-eoe_072007_eng.pdf. Accessed May 9, 2017.
2. Uganda Ministry of Health, 2012. *Guidelines for Certification of Onchocerciasis Elimination in Uganda*. Kampala, Uganda: Uganda Ministry of Health.
3. World Health Organization, 2001. *Certification of Elimination of Human Onchocerciasis: Criteria and Procedures*. WHO Document WHO/CDS/CPE/CEE/2001.18a. Geneva: WHO Press.
4. World Health Organization, 2016. *Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis: Criteria and Procedures*. WHO Document WHO/HTM/NTD/PCT/2016.1. Geneva, Switzerland: WHO Press.
5. Toe L, Merriweather A, Unnasch TR, 1994. DNA probe based classification of *Simulium damnosum* s.l. borne and human derived filarial parasites in the Onchocerciasis Control Programme area. *Am J Trop Med Hyg* 51: 676–683.
6. Katholi CR, Toe 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.
7. Fischer P, Yocha J, Rubaale T, Garms R, 1997. PCR and DNA hybridization indicate the absence of animal filariae from vectors of *Onchocerca volvulus* in Uganda. *J Parasitol* 83: 1030–1034.
8. Walsh JF, Davies JB, LeBerre R, Garms R, 1978. Standardization of criteria for assessing the effects of *Simulium* control in onchocerciasis control programmes. *Trans R Soc Trop Med Hyg* 72: 675–676.
9. Keddie EM, Higazi T, Unnasch TR, 1998. The mitochondrial genome of *Onchocerca volvulus*: Sequence, structure and phylogenetic analysis. *Mol Biochem Parasitol* 95: 111–127.
10. Casiraghi M, Anderson TJ, Bandi C, Bazzocchi C, Genchi C, 2001. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasitology* 122: 93–103.
11. Kumar S, Stecher G, Tamura K, 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33: 1870–1874.
12. Lefoulon E, Giannelli A, Makepeace BL, Mutafovchiev Y, Townson S, Uni S, Verocai GG, Otranto D, Martin C, 2017. Whence river blindness? The domestication of mammals and host-parasite co-evolution in the nematode genus *Onchocerca*. *Int J Parasitol* 47: 457–470.
13. de Jong YA, Cumming D, d'Huart J, Butynski T, 2016. *Pha-cochoerus africanus*. Available at: <http://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T41768A44140445.en>. Accessed May 3, 2017.
14. Lamberton PH et al., 2016. Onchocerciasis transmission in Ghana: the human blood index of sibling species of the *Simulium damnosum* complex. *Parasit Vectors* 9: 432.
15. Eisenbarth A, Achukwi MD, Renz A, 2016. Ongoing transmission of *Onchocerca volvulus* after 25 years of annual ivermectin mass treatments in the Vina du Nord River Valley, in North Cameroon. *PLoS Negl Trop Dis* 10: e0004392.