Duplex Real-Time PCR Assay for Clinical Differentiation of *Onchocerca lupi* and *Onchocerca volvulus*

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Abstract. In the United States and Europe, human onchocerciasis is a rare disease caused by zoonotic or anthropophilic parasites in the genus *Onchocerca*. The zoonotic species identified in focal areas of Europe and United States is *Onchocerca lupi*, and *Onchocerca volvulus*, the anthroponotic species, may be found among people who had lived in endemic areas of Africa, the Arabian Peninsula, or Latin America. Onchocerciasis due to *O. lupi* is an emergent parasitic disease, with limited diagnostic methods, in addition to the lack of information on its biology, transmission, and epidemiology. Cutaneous nodules are the disease's most prevalent manifestation but lack diagnostic specificity. To address the diagnosis of onchocerciasis at reference laboratories, we developed a duplex TaqMan real-time PCR (qPCR) method, targeting the cytochrome oxidase subunit I locus which has species-specific probes to identify and differentiate *O. lupi* from *O. volvulus*. We determined the performance of the duplex with a panel of 45 samples: 11 positives for *O. lupi*, six for *O. volvulus*, five samples with negative results for *Onchocerca* spp., and 23 non-*Onchocerca* nematodes. The duplex qPCR correctly detected 10 of 11 *O. lupi*– and six of six *O. volvulus*–positive specimens. The new duplex assay allowed the simultaneous detection and discrimination of *O. lupi* and *O. volvulus* in clinical specimens, expediting and facilitating the clinical diagnosis of *O. lupi* in non-endemic settings where the disease is an infrequent finding.

INTRODUCTION

Human onchocerciasis is caused by zoonotic and anthropophilic species parasites in the genus *Onchocerca*, including *Onchocerca lupi* which is a zoonotic species. It was first described in 1967 from the eye of a wolf in Georgia. Since then, *O. lupi* infections have been reported in at least 90 dog and 22 human cases in focal areas of Europe and North America.[1,2] Other veterinary *Onchocerca* species such as *Onchocerca gutturosa*, *Onchocerca cervicalis*, *Onchocerca jakutensis*, and *Onchocerca dewittei japonica* have also been infrequently implicated in human onchocerciasis.[3–7] *Onchocerca volvulus*, the anthropophilic species transmitted by black flies of the genus *Simulium*, causes human onchocerciasis, also known as river blindness. An estimated 21 million people worldwide are infected,[8] with approximately 205 million people[9] living at risk of infection in focal endemic areas of Africa, the Arabian Peninsula, and Latin America.[10–12] Human migration has been the main source of epidemics throughout, facilitating emergence of new infections and changing the distribution, severity, and frequency of known infections.[13] Given the great increase in international and adventure travel over the past four decades, for epidemiological and clinical diagnostic perspectives, reference laboratories may have to consider the use of standardized methods that allow simultaneous detection and differentiation of these parasites.

In humans, *O. lupi* has particular characteristics not commonly observed in other zoonotic filarial infections, such as the presence of multiple worms in a single patient including gravid females.[1] Cutaneous/subcutaneous nodules are the most common clinical manifestations of *O. lupi* infection; however, cervical spine involvement has been associated with a few cases.[1,2,14] Spinal onchocercoma manifestations are not observed in *O. volvulus*, where the primary manifestations are severe itching, depigmentation or fibrosis of the skin, and progressive ocular lesions that could lead to partial or total visual impairment.[11,15] *Onchocerca lupi* is an emerging parasite, and there is an increasing interest to fully understand its biology, transmission vectors, geographical distribution, epizootiology in dogs, identification of other potential reservoirs, and human epidemiology.

Although serologic methods are used for monitoring *O. volvulus* infection in control and elimination programs, examination of skin snip biopsies is used for the clinical diagnosis of human onchocerciasis.[10,16–20] Species-level identification of larvae by microscopy is performed only by well-trained staff, requiring appropriate preservation and processing of specimens to ensure that morphologic features are not lost. Adult *Onchocerca* spp. can be identified by histopathology of nodules, and the species identification relies on features of the multilayered cuticle such as annular ridges (on the external layer) and transverse striae (on the internal layer), as well as size of the worm, appearance of the somatic muscles, size of the lateral chords, and structure of the vagina.[10,18–20]

In comparison with microscopy, molecular techniques are often more sensitive and specific for the detection of positive cases, especially when the clinical specimens have low parasite density, or the morphologic features have been degraded during processing.[16,21–23] Detection of *O. volvulus* by conventional PCR relies most often on amplification of O-150 locus, a highly polymorphic DNA segment with approximately 4,500 copies per *Onchocerca* sp. genome.[24,25] Amplification of 12S ribosomal RNA genes (rRNA) and cytochrome oxidase subunit I (COX-I) has also been successfully used for confirmatory detection of *Onchocerca* species.[5,17,26–28] Molecular diagnosis of onchocerciasis is increasingly relying on real-time PCR (qPCR) because it is a highly sensitive technique, and is faster than conventional PCR followed by DNA sequence analysis. It also minimizes cross contamination and provides results in a manner which facilitates data analysis. The qPCR assays used for identification of *Onchocerca* species have shown improved diagnostic performance when compared with conventional PCR methods.[23,29–31]

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In our laboratory, specimens from suspected cases of onchocerciasis are first examined by microscopy, which is followed by sequencing the products of PCR amplification of the mitochondrial 12S and 16S rRNA loci. However, this methodology is time consuming, and may sometimes provide inconclusive results depending on the specimen tested. To address this limitation and provide a new alternative, we modified a qPCR TaqMan method for *O. lupi*.[29] The modified assay used newly designed genus-specific primers and two species-specific TaqMan probes targeting the COX-I locus, to simultaneously detect and differentiate *O. lupi* from *O. volvulus*. The results of the duplex qPCR were compared with microscopy and conventional PCR, showing that this assay could expedite the differential diagnosis of onchocerciasis in reference laboratories.

MATERIALS AND METHODS

Panel of specimens tested. The performance of the *O. lupi*/*O. volvulus* duplex qPCR assay was evaluated using 45 DNA samples from 11 clinical specimens collected from nine patients positive for *O. lupi*, adult worms isolated from six clinical cases with positives results for *O. volvulus*, five human tissue biopsies submitted for *O. lupi* diagnosis with negative results, 11 specimens from noninfected people, and 12 from other helminths: *Wuchereria bancrofti*, *Brugia malayi*, *Dirofilaria immitis*, *Trichinella nativa*, *Trichinella spiralis*, *Trichinella murrelli*, *Trichinella* T6, *Anisakis* sp., *Paragonimus mexicanus*, *Trichuris* sp., *Taenia solium*, and *Halicephalobus gingivalis*. In addition, DNA-extracted adult worms previously identified as *O. lupi* and *O. volvulus* by microscopy were used as qPCR assay reference controls. Details on the geographic origin and date of collection, microscopic parasite identification, and the type of samples used for DNA extractions are presented in Table 1.

DNA extraction. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), following the manufacturer's instructions,[32,33] using either 10–20 mg of human biopsies, 0.2 mL of blood, or worm sections of 1–10 mm in length.

Oligonucleotides design. Oligonucleotide primers were designed for conventional PCR, DNA sequence analysis, and qPCR amplification.

Conventional PCR. We designed genus-level primers targeting the mitochondrial 12S and 16S rRNA genes. Primers for the 12S locus Onch12SF/Onch12SR were designed using sequences of *O. lupi* GenBank (accession no. KC763784), *O. volvulus* (accession no. AY462920), *O. gutturosa* (accession no. AY462923), *O. jakutensis* (accession no. HQ717719), and *O. dewittei japonica* (accession no. AM779814). The primers Onch16SF/Onch16SR amplified the 16S locus and were based on sequences from *O. volvulus* (accession no. AY462903) and *O. gutturosa* (accession no. AY462904). These primers were used for our in-house conventional PCR designed to amplify fragments of 270 and 430 bp of the 12S and 16S rRNA, respectively.

TaqMan qPCR. Sequences of COX-I gene of *Onchocerca* species, including *O. lupi* (accession nos. JX080028 and HQ207644), *O. volvulus* (accession no. AM749285), *O. gutturosa* (accession no. KP760201), *O. dewittei japonica* (accession no. KP760203), *Onchocerca lienalis* (accession no. KX853325), *Onchocerca fasciata* (accession no. JQ316672), *Onchocerca flexuosa* (accession no. HQ214004), and *Onchocerca ochengi* (accession no. KX181289), were used as references for the design of primers and probes of the duplex qPCR assay. We designed primers and probes to amplify and detect a fragment of the COX-1 gene previously reported.[29] Primers ONCOXI-F and ONCOXI-R were designed to be genus specific and amplify products of approximately 135 bp. ONCOXI-F was designed with two degenerate nucleotides (nt9-R and nt12-Y) to amplify both *O. lupi* and *O. volvulus*. The species-specific probes were *O. lupi* OLCOXI-PB and *O. volvulus* OVCOXI-PB, labeled with the FAM (fluorescein-amidite; 6-carboxyfluorescein) and VIC (aequorea victoria green fluorescent protein; 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein) fluorophores, respectively. Oligonucleotide and probe sequences are listed in Table 2.

***Onchocerca lupi*/*O. volvulus* duplex qPCR assay.** The duplex qPCR protocol was optimized as described elsewhere.[29] The reactions were prepared with 0.25 µM of each primer, 0.15 µM of each probe, 12.5 µL of TaqMan® Universal PCR Master Mix (Applied Biosystems, Waltham, MA), 3 µL of DNA template, and sterile water to adjust the volume to 25 µL. PCR was performed on ABI 7500 real-time PCR system (Applied Biosystems) using the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence data were collected at the end of each 60°C plateau.

Reproducibility and evaluation of limit of detection (LOD) of *O. lupi*/*O. volvulus* duplex qPCR assay and specimen's quality control. The reproducibility of duplex qPCR was verified by testing all samples at least on two separate runs, on different dates. The LOD was performed in reactions prepared with mixed *O. lupi*/*O. volvulus* DNA extracted from worms of both species in 10-fold dilutions ranging from 3.0 to 3×10^{-12} µg/µL. Internal quality control for DNA integrity was based on the SYBR green detection of a fragment of the human β-actin gene, as previously described.[34]

Conventional PCR and Sanger DNA sequencing analysis. 12S and 16S PCR reactions (12SPCR and 16SPCR) were prepared with 0.2 µM each of 16S or 12S primers, 45 µL of Platinum Blue PCR SuperMix mixture (Invitrogen, Waltham, MA), and 3 µL of DNA. The total volume of the PCR reactions was 50 µL. The amplification protocol was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. The amplicons were purified with StrataPrep PCR purification kit (Stratagene, La Jolla, CA) and subjected to Sanger sequencing using BigDye version 3.1 chemistry (Applied Biosystems) following the manufacturer's instructions. Conventional PCR amplification and cycle sequencing were carried out in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems). Amplification products were visualized by gel electrophoresis.

The amplified reaction mixtures were purified through DyeEx 2.0 Spin Kit (Qiagen) and analyzed on an ABI Prism 3130XL sequence analyzer, with data collection software, V3.0 (Applied Biosystems). The sequences were assembled, edited, and aligned in DNA STAR SeqMan v. 14.0.0 (88) 422 (DNASTAR Inc., Madison, WI) software.

Next-generation sequencing (NGS). Amplicon deep sequencing was used to obtain DNA sequences from samples that had yielded unreadable Sanger sequences (overlapping peaks in the electropherograms). Those samples were re-amplified in triplicates and DNA libraries prepared using NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA) as per manufacturer's instructions for 1 ng of DNA input. The libraries were quantified using a Qubit fluorometer 2.0 with dsDNA HS assay kit (Invitrogen-Thermo

TABLE 1
List of specimens investigated using the *O. lupi*/*O. volvulus* real-time PCR duplex assay

Patient number	DNA number	Parasite	Origin,* year	Sample type‡	Additional information‡
1	1	*O. lupi*	AZ, 2014	FFPE	Human nodule biopsy†
2	2	*O. lupi*	TX/NM/CO, 2014	FFPE	Human nodule biopsy†
	3	*O. lupi*	TX/NM/CO, 2014	FFPE	Human nodule biopsy†
3	4	*O. lupi*	AZ, 2014	FFPE	Human nodule biopsy†
4	5	*O. lupi*	NM/CO, 2014	FFPE	Human nodule biopsy†
5	6	*O. lupi*	AZ/UT, 2014	FFPE	Human nodule biopsy†
2	7	*O. lupi*	TX/NM/CO, 2014	FFPE	Human nodule biopsy†
6	8	*O. lupi*	NM, 2019	FFPE	Human nodule biopsy†
7	9	*O. lupi*	NM, 2019	FFPE	Human nodule biopsy†
8	10	*O. lupi*	AZ, 2017	FFPE	Human nodule biopsy†
9	11	*O. lupi*	TX, 2014	Worm	–
10	12	*O. volvulus*	Chad, NA	Worm	Adult *O. volvulus*†
11	13	*O. volvulus*	Chad, NA	Worm	Adult *O. volvulus*†
12	14	*O. volvulus*	Chad, NA	Worm	Adult *O. volvulus*†
13	15	*O. volvulus*	DRC, NA	Worm	Adult *O. volvulus*†
14	16	*O. volvulus*	Ethiopia, NA	Worm	Adult *O. volvulus*†
15	17	*O. volvulus*	S. Sudan, NA	Worm	Adult *O. volvulus*†
16	18	Negative	AZ, 2015	Fresh tissue	Human nodule biopsy
17	19	Negative	French Polynesia, 2016	Blood	Human blood
18	20	Negative	S. Sudan, 2017	FFPE	Human nodule biopsy
	21	Negative	S. Sudan, 2017	FFPE	Human nodule biopsy
	22	Negative	S. Sudan, 2017	FFPE	Human nodule biopsy
NA	23–33	Negative human DNA	USA, 2019	Fresh tissue	Human skin biopsy
NA	34	*Wuchereria bancrofti*	CDC collection	FFPE	Filaria, worm†
NA	35	*Brugia malayi*	CDC collection	FFPE	Filaria, worm†
NA	36	*Dirofilaria immitis*	CDC collection	Fresh tissue	Filaria, adult worm
NA	37	*Trichuris* sp.	CDC collection	Fresh tissue	Nematode, adult worm
NA	38	*Trichinella nativa*	CDC collection	Fresh tissue	Nematode, larvae
NA	39	*Trichinella spiralis*	CDC collection	Fresh tissue	Nematode, larvae
NA	40	*Trichinella murrelli*	CDC collection	Fresh tissue	Nematode, larvae
NA	41	*Trichinella* T6	CDC collection	Fresh tissue	Nematode, larvae
NA	42	*Anisakis simplex*	CDC collection	Fresh tissue	Nematode, larva
NA	43	*Taenia solium*	CDC collection	FFPE	Cestode, tapeworm
NA	44	*Paragonimus mexicanus*	CDC collection	FFPE	Trematode, adult worm
NA	45	*Halicephalobus gingivalis*	CDC collection	FFPE	Nematode-free living

FFPE = formalin-fixed paraffin-embedded; *O. lupi* = *Onchocerca lupi*; *O. volvulus* = *Onchocerca volvulus*. Twenty-two samples from the United States and Africa characterized by microscopy analysis and 23 non–*Onchocerca*-related specimens from the United States.

* AZ = Arizona; TX = Texas; NM = New Mexico; CO = Colorado; UT = Utah; NA = not applicable. Specimens 2, 3, and 7 are from a patient from Texas with a travel history to New Mexico and Colorado; specimen 5 is from a patient from New Mexico with a travel history to Colorado; specimen 6 is from a patient from Arizona with a travel history to Utah.

† Nodule biopsies (fresh or FFPE samples) may have included adult worms and microfilariae.

‡ DNA 1–22 were obtained from 18 suspect onchocerciasis clinical cases, and DNA 2, 3, and 7 and DNA 20, 21, and 22 were extracted from two patients. DNA 23–33 were obtained from non–helminths-related clinical cases.

Fisher, Waltham, MA), and the peak sizes were assessed with TapeStation using D1000 DNA Screen Tape (Agilent, Santa Clara, CA). The barcoded libraries were diluted to 4 nM, pooled, and 15 PM were loaded in a MiSeq Kit 2 × 250 (Illumina, San Diego, CA). The resulting reads were imported into CLC Genomics workbench 11.0 for trimming (quality and adapters) and mapping. The consensus sequences from each of those products were queried in GenBank using the BLASTn tool. We used results which had at least 97% identity to the sequences previously used for oligonucleotide design.

RESULTS

Microscopy analysis. The characterization of 22 samples, including 17 suspected cases of onchocerciasis, was performed on the basis of morphological features, that is, the thickness of the cuticle, presence or absence of outer cuticular ridges, and the position of the inner cuticular striae. Based on these characteristics, four worm specimens from three patients were identified as *O. lupi*, and samples isolated from six cases were identified as *O. volvulus*. Seven *Onchocerca* adults of unknown species were identified in another six clinical

TABLE 2
Oligonucleotides used in the preparation of the multiplex real-time PCR assay and 16S/12S conventional PCR

Name	Oligonucleotide sequences (5′–3′)	Reference sequence	Target gene (nt)
ONCOXI-F	TTT ATT GGR GGY GGT CCT GGT A	JX080028	COX-I (110–131)
ONCOXI-R	TAA TAG CAC CCA ACA AAG AAC C	JX080028	COX-I (204–245)
OLCOXI-PB	FAM - TAG AGT AGA GGG TCA GCC TGA GTT ATC- BHQ1	JX080028	COX-I (157–182)
OVCOX I-PB	HEX - AGG GTT GAA GGT CAA CCA GAA TTG TC- BHQ1	AM749285	COX-I (158–182)
Oncho 12S-F	TTT GAA CTG GAT TAG TAC CCA G	KC763784	rRNA 12S (146–167)
Oncho 12S-R	AAA AAT TTA TAA TAG TAA CAC ATG	KC763784	rRNA 12S (413–390)
Olupi16SF	GAT GGC ATA AAA GTA GCG TAA GTG A	AY462903	rRNA 16S (12–36)
Olupi16SR	AAG ATA AAC CGC TCT GTC TCA CG	AY462903	rRNA 16S (435–413)

COX-I = cytochrome oxidase subunit I; *O. lupi* = *Onchocerca lupi*.

samples, and five specimens from three cases were found to be negative for *Onchocerca* species (Table 3).

Quality control. All the 45 DNA extracts had positive amplification of the β-actin locus by SYBR Green qPCR, indicating a lack of PCR inhibitors in the extracted DNA. However, sample 4 was of limited availability for the DNA extraction and yields β-actin gene SYBR Green cycle threshold (Ct) values of 37.5. Compared with the average Ct values among the other *Onchocerca* spp.–positive samples (≤ 23.2), the high Ct value of sample 4 was indicative of very low DNA concentration in the sample (Table 3).

Performance of *O. lupi/O. volvulus* duplex qPCR. The performance of the duplex qPCR assay for the discrimination

of *O. lupi* and *O. volvulus* was verified using the suspected 22 onchocerciasis cases submitted to our laboratory for diagnostic confirmation by microscopy. All clinical samples were also analyzed by conventional PCR and sequencing of the 12S and 16S loci (samples 1–22, Table 1). From the 22 clinical samples, five samples were negative by all tests. From the remaining 17 specimens, 16 were positive by microscopy and PCR: 11 were *O. lupi* and six were *O. volvulus*. One of the 17 specimens (4) was microscopy positive for *O. lupi* but negative by PCR.

The duplex qPCR consistently amplified those 16 positive samples and correctly identified either *O. lupi* or *O. volvulus*. The 11 samples with *O. lupi* had a single FAM fluorescent signal with Ct values ranging from 18 to 35. The six samples

TABLE 3
Summary of results obtained from the *O. lupi/O. volvulus* biplex qPCR assay, conventional PCR, followed by DNA sequencing and microscopy analysis

| Specimen number | Type | Biplex qPCR | | | Conventional PCR followed by DNA sequencing† | | Microscopy |
		O. lupi Ct value (FAM)	*O. volvulus* Ct value (HEX)	Internal control Ct value	12S PCR Sequencing	16S PCR Sequencing	
1	FFPE	29.02	Negative	21.6	*O. lupi*	*O. lupi*	*O. lupi*
2	FFPE	30.35	Negative	22.2	*O. lupi*	*O. lupi*	*O. lupi*
3	FFPE	33.53	Negative	21.9	*O. lupi**†	Weak*‡	*O. lupi*
4	Fresh tissue	Negative	Negative	37.5	Negative	Negative	*O. lupi*
5	FFPE	28.38	Negative	23.2	*O. lupi**†	*O. lupi**†	*Onchocerca* sp.
6	FFPE	35.03	Negative	21.8	Weak*‡	*O. lupi**†	*Onchocerca* sp.
7	FFPE	30.37	Negative	21.5	*O. lupi*	*O. lupi*	*Onchocerca* sp.
8	FFPE	30.43	Negative	22.2	Weak*‡	*O. lupi*	*Onchocerca* sp.
9	FFPE	31.37	Negative	22.3	Weak*‡	*O. lupi*	*Onchocerca* sp.
10	FFPE	27.37	Negative	21.4	*O. lupi*	Weak*‡	*Onchocerca* sp.
11	Worm	18.58	Negative	22.3	*O. lupi*	*O. lupi*	*Onchocerca* sp.
12	Worm	Negative	25.27	20.7	*O. volvulus**†	*O. volvulus**†	*O. volvulus*
13	Worm	Negative	20.39	21.3	*O. volvulus*	*O. volvulus*	*O. volvulus*
14	Worm	Negative	22.22	21.0	*O. volvulus*	*O. volvulus*	*O. volvulus*
15	Worm	Negative	20.20	21.0	*O. volvulus*	*O. volvulus*	*O. volvulus*
16	Worm	Negative	24.28	22.0	*O. volvulus*	*O. volvulus*	*O. volvulus*
17	Worm	Negative	14.99	21.9	*O. volvulus*	*O. volvulus*	*O. volvulus*
18	Fresh tissue	Negative	Negative	21.3	Negative	Negative	Negative
19	Blood	Negative	Negative	21.1	Negative	Negative	Negative
20	FFPE	Negative	Negative	22.0	Negative	Negative	Negative
21	FFPE	Negative	Negative	21.8	Negative	Negative	Negative
22	FFPE	Negative	Negative	26.8	Negative	Negative	Negative
23	Human DNA	Negative	Negative	24.8	NA	NA	NA
24	Human DNA	Negative	Negative	22.1	NA	NA	NA
25	Human DNA	Negative	Negative	26.4	NA	NA	NA
26	Human DNA	Negative	Negative	20.6	NA	NA	NA
27	Human DNA	Negative	Negative	24.5	NA	NA	NA
28	Human DNA	Negative	Negative	25.7	NA	NA	NA
29	Human DNA	Negative	Negative	27.9	NA	NA	NA
30	Human DNA	Negative	Negative	25.9	NA	NA	NA
31	Human DNA	Negative	Negative	20.4	NA	NA	NA
32	Human DNA	Negative	Negative	20.9	NA	NA	NA
33	Human DNA	Negative	Negative	26.9	NA	NA	NA
34	*Wuchereria bancrofti*	Negative	Negative	29.7	NA	NA	NA
35	*Brugia malayi*	Negative	Negative	34.3	NA	NA	NA
37	*Dirofilaria immitis*	Negative	Negative	33.8	NA	NA	NA
37	*Trichuris* sp.	Negative	Negative	30.8	NA	NA	NA
38	*Trichinella nativa*	Negative	Negative	32.4	NA	NA	NA
39	*Trichinella spiralis*	Negative	Negative	21.3	NA	NA	NA
40	*Trichinella murrelli*	Negative	Negative	25.6	NA	NA	NA
41	*Trichinella* T6	Negative	Negative	24.3	NA	NA	NA
42	*Anisakis simplex*	Negative	Negative	20.6	NA	NA	NA
43	*Taenia solium*	Negative	Negative	31.4	NA	NA	NA
44	*Paragonimus mexicanus*	Negative	Negative	23.6	NA	NA	NA
45	*Halicephalobus gingivalis*	Negative	Negative	32.3	NA	NA	NA

FFPE = formalin-fixed paraffin-embedded; *O. lupi = Onchocerca lupi*; *O. volvulus = Onchocerca volvulus*; qPCR = real-time PCR. SYBR Green qPCR internal control (Ct values) represents the quality of DNA aliquots.
* DNA aliquots weakly amplified.
† Species identified by using partial Sanger sequencing and/or next-generation sequencing.
‡ Failed to provide sequencing data.

positive for *O. volvulus* had a single VIC fluorescent signal, with Ct values ranging from 15 to 25. No fluorescent signal was detected in the five negative samples (18–22). The real-time and 12S and 16S conventional PCR failed to amplify specimen 4. The molecular test results were 100% concordant between the conventional and qPCR, with an overall sensitivity of 91% (10/11), for *O. lupi* and 100% (*n* = 6) for *O. volvulus*.

The duplex qPCR specificity was 100%, as shown by the negative amplification of human samples and DNA extracted from other helminths (samples 23–45). The LOD of the duplex qPCR was calculated to be < 3 × 10^{-6} ng/µL. Results are summarized in Table 3.

Species-specific identification of *O. lupi* and *O. volvulus* using conventional PCR followed by DNA sequencing analysis. *12S PCR.* Sixteen of the 22 samples tested by microscopy had positive amplification (nine nodule biopsies and seven worms), including five clinical specimens weakly amplified (3, 5, 6, 8, and 9). The sequencing data of these amplicons were compared with the GenBank database allowing the identification of seven *O. lupi* cases, including two identified only with partial sequences (specimens 3 and 5) and six *O. volvulus* cases. Three amplicons (specimens 6, 8, and 9) failed to provide sequences.

16S PCR. To verify species identification, the 22 samples were further analyzed at the 16S locus. All 16 samples with positive 12S amplification were positive at the 16S locus and subjected to DNA sequencing, including five samples with weak amplification products (3, 5, 6, 10, and 12). Six samples (12–17) had > 99% of identity to *O. volvulus*. Of the remaining 10 samples, nine had highly homologous sequences, showing 94% identity with *O. flexuosa* and *O. lienalis* (accession nos. HQ214004 and AY462906), respectively, in the GenBank database. Because GenBank did not have sequences for *O. lupi* at this locus, we submitted the consensus sequence from specimens 1 and 2 (accession no. KM347895). One sample (specimen 10), failed to provide sequences. In addition, specimen 4, previously diagnosed as positive for *O. lupi* by microscopy, did not amplify at either the 16S or 12S loci. The five samples (18–22) previously diagnosed negative for *Onchocerca* spp. by microscopy were also negative at both 16S and 12S loci. Results are summarized in Table 3.

Next-generation sequencing for species verification of 12S and 16S amplicons. We used NGS to obtain complete sequences from seven samples that weakly amplified at the 12S and/or 16S loci. Specimens 3, 5, 6, and 12 provided enough amplified DNA for NGS analysis, whereas amplification of specimens 8, 9, and 10 failed at both loci. The NGS data were generated from mapping sequences with ×100 coverage on average.

12S. The amplicon sequencing of specimen 12 had 99% similarity with *O. volvulus* (accession no. AY462920). The sequences from nodule biopsies 3 and 5 were homologues, with 100% identity with *O. lupi* (accession no. KC763784) reference. Specimen 6 did not yield enough NGS reads for analysis.

16S. The consensus sequence of specimen 12 was > 99% similar to *O. volvulus* (accession no. AY462903). The sequences of specimens 5 and 6 had > 99% similarity with *O. lupi* (accession no. KM347895). Specimen 3 did not yield NGS data.

DISCUSSION

Human onchocerciasis in non-endemic settings is an infrequent finding, and it has usually been linked to people who

lived for extended periods in endemic areas. However, *O. lupi* has recently emerged in Tunisia, Iran, Greece, Germany, and the United States, areas not endemic for *O. volvulus*.[5,20,22,27,35] Although the geographic separation of *O. lupi* and *O. volvulus* is evident, migrant populations or travelers can harbor many infections contributing to introduction, adaptation, and circulation of pathogenic microbes into new geographic areas. Depending on the presence of vectors and the hosts' susceptibility, this factor contributes to the emergence of infectious diseases, increasing the public health implications, rates of transmission, and infectivity.[13] Therefore, because infections by *O. lupi* may have more severe clinical manifestations than *O. volvulus*, including cervical spine involvement, reference laboratories must provide species-level identification of *Onchocerca* spp. to properly assist in decisions for medical treatment. In addition, accurate parasite identification provides a better understanding on the distribution and prevalence of the disease, especially in patients who may have an extensive travel history.[19,36,37]

In our laboratory, suspected cases of onchocerciasis are investigated using both microscopy and PCR, followed by DNA sequencing analysis. Because the conventional PCR methods were designed using generic *Onchocerca* primers, they can theoretically amplify any *Onchocerca* parasite, and DNA sequence analysis could determine the species as either *O. lupi* or *O. volvulus*. However, as demonstrated in this study, the suboptimal amplification of some specimens may not allow species identification by DNA sequencing. Consequently, we developed a new TaqMan qPCR assay targeting the COX-I locus for the detection and specific identification of *O. lupi* and *O. volvulus* in clinical specimens. This novel qPCR assay was adapted from the method described by Latrofa et al.,[29] into the duplex assay format using one primer pair and species-specific TaqMan probes for *O. lupi* and *O. volvulus*.

Although microscopic analysis is the most common technique used for the diagnosis of infection with *O. lupi* and *O. volvulus*, the sensitivity and specificity of microscopy depends on the quality of the specimen and the microscopy skills of the staff tasked with identifying parasites. DNA-based detection methods are more sensitive methods to detect *Onchocerca* spp.[38–40]; however, the efficiency of these methods (specificity and sensitivity) depends on the quality of the preserved specimen and DNA template, set of oligonucleotides selected, and the size of fragments amplified. For instance, the O-150 locus, the standard target used for the detection of *O. volvulus* in programs for monitoring and elimination of onchocerciasis, has intraspecies variation that can affect the sensitivity of PCR methods.[23] The rRNA and COX-I genes are multi-copy genes with low rates of polymorphism, providing species-specific results to distinguish *Onchocerca* spp. by multiplex qPCR assays.[21,29,38–41]

The performance of the new qPCR in this study demonstrated good detection limits and did not cross-react with human DNA or other helminths tested. The results of the qPCR were in agreement with 12S and 16S PCR results and showed 95% (20/21) agreement with microscopic analysis. It should be noted that the molecular methods missed one case (specimen 4) that was microscopy positive for *O. lupi*. We attributed the discrepant results to the low concentration of total DNA obtained from the very small amount of clinical sample available for DNA extraction (approximately 1 mm of tissue). This hypothesis is supported by the weak amplification of this DNA aliquot using the SYBR qPCR (Ct. 37.5). This finding

highlights the importance of adequate amount and preservation of the specimen to be tested. The analytical sensitivity or LOD of the duplex qPCR was similar to that of single target assays reported in other studies.[29,32] Although the detection of *O. lupi* and *O. volvulus* was concordant with PCR and sequencing results, the qPCR allowed the species-specific identification of these parasites, providing faster and simultaneous identification, including in those cases that provided only partial or no sequencing data, or cases identified only to the genus level by microscopy. It should be emphasized that the efficiency of PCR assays may be affected by aforementioned factors. Samples poorly preserved will lead to substandard amplification, possibly because of DNA denaturation, or by the presence of PCR inhibitors which can be found in DNA extracts from FFPE.[42]

CONCLUSION

The duplex qPCR method allowed the identification of *O. lupi* while differentiating from *O. volvulus*. This duplex qPCR TaqMan improved and streamlined the clinical diagnosis of *O. lupi* onchocerciasis in a U.S. reference laboratory, showing advantages over cPCR and DNA sequencing.

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