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Enhanced molecular diagnosis of Onchocerca lupi using droplet digital PCR in clinically suspected companion animals

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Abstract

Background

Onchocerca lupi, a zoonotic filarial nematode documented in southwestern North America and the Old World, is associated with ocular onchocerciosis in dogs and cats. Clinical signs range from ocular discharge and conjunctivitis to chronic nodular lesions involving the conjunctiva, sclera, and retrobulbar space. Many infections remain subclinical and undiagnosed. Current diagnostic approaches rely on microscopy and conventional PCR (cPCR) to confirm adult worms in nodules or microfilariae in skin snips. Real-time PCR (qPCR) has been applied primarily in large-scale epidemiological studies. However, amplification of *O. lupi* DNA may be inhibited, leading to false-negative results. Highly sensitive techniques such as droplet digital PCR (ddPCR) can enable the detection of parasite DNA in various biological samples through absolute quantification. The objectives of this study were to: I) validate a novel ddPCR assay that detects *O. lupi*, and II) assess the performance of the ddPCR assay compared to cPCR and qPCR in suspected clinical cases.

Methods

Following assay validation and optimization, 202 suspected clinical cases from 11 US states were assessed. Of these clinical cases, 97% (n = 196/202) were dogs, and 3% (n = 6/202) were cats. Samples included adult specimen *O. lupi* fragments (n = 34/202), subconjunctival nodule biopsies (n = 103/202), interscapular skin snips (n = 23/202), lower ear skin snips (n = 3/202), and formalin-fixed paraffin-embedded (FFPE) tissue samples (n = 39/202), all previously assessed using cPCR and qPCR. Statistical analysis was performed to assess agreement using Cohen's kappa (κ), and Cochran's Q test was used to compare the pattern of positive/negative results of the three diagnostic techniques.

Results

Overall, the ddPCR detected 71.3% (n = 144/202) of clinical cases as positive for *O. lupi*, including 71.9% of dog samples (n = 141/196), outperforming qPCR (64.8%; n = 127/196) and cPCR (54.1%; n = 106/196). Additionally, we confirmed *O. lupi* infection in 50% (n = 3/6) of the cat samples across all three diagnostic tests. Statistical analysis showed moderate agreement between cPCR + qPCR (κ = 0.74), fair agreement between cPCR + ddPCR (κ = 0.60), and almost perfect agreement between qPCR + ddPCR (κ = 0.81). This study represents the largest series of suspected *O. lupi* clinical cases in companion animals in the US to date, with positive detections reported in 10 of 11 sampled states, although travel history was not always available.

Conclusion

The validated, host-agnostic ddPCR assay demonstrated superior sensitivity across multiple sample types and provides a robust tool for clinical diagnosis and large-scale surveillance studies, supporting improved epidemiological understanding and One Health-based prevention strategies.

Introduction

Onchocerca lupi (Filarioidea, Onchocercidae) is an emerging zoonotic filarioid nematode that is the causative agent of ocular onchocerciosis in companion animals [1–3]. Originally described from a grey wolf, *O. lupi* has been documented in the Old World, specifically Africa, Asia, Europe, as well as North America [4–10]. Several knowledge gaps exist for this filarial nematode across biological factors, such as the pre-patent period and vectorial competence, as well as epidemiological factors, including baseline prevalence in endemic areas, particularly in the southwestern United States (US), and the establishment of local transmission in non-endemic areas due to the translocation of animals. Although several case series and epidemiological studies have documented the low prevalence of *O. lupi* in the US, further research is needed to understand this zoonotic filarial nematode better [11–16].

For this cutaneous dwelling filarial nematode, canids are considered the definitive hosts, with dogs (*Canis lupus familiaris*) the most frequently reported, followed by coyotes (*Canis latrans*), and wolves (*Canis lupus*) reported the least [11–13, 16–26]. The most extensive case series in the US on canine *O. lupi* to date comprised 16 clinical cases and summarized diagnostics and treatment strategies [11]. In addition, a few epidemiological surveillance studies have been conducted in various southwestern US states,

including New Mexico, Arizona, and Utah, to estimate baseline prevalence [14–16]. Feline infections are rare, with only four confirmed cases worldwide, including two in the US, one in Portugal, and one in Romania [20, 27, 28]. Zoonotic infections have also been documented, with nine human cases confirmed in the US to date, with the majority originating from southwestern states [29–36].

Until recently, the intermediate host of *O. lupi* was unknown [5, 37]. Emerging molecular evidence indicates that both black flies (*Simulium tribulatum*) and biting midges (Ceratopogonidae sp.) may serve as competent intermediate hosts. *Onchocerca lupi* DNA has been detected in pools of black flies collected in southern California [38] and in the heads of biting midges from the Navajo Reservation in the southwestern US [15], supporting their potential role in parasite transmission.

Clinically, ocular *O. lupi* infection in companion animals can present in one or both eyes, ranging from acute manifestations such as conjunctivitis and ocular discharge to chronic stages characterized by nodular development in various parts of the eye, with the potential for enucleation [11]. However, many cases go undiagnosed due to the subclinical nature of the infections or nonspecific clinical signs. Currently, no standardized or consistently effective treatment protocol for *O. lupi* has been established. Several case series indicate that a combination of surgical excision and drug therapy (i.e., ivermectin, doxycycline, prednisone) may be effective, as observed in other filarial nematode infections, such as *Dirofilaria immitis* [11, 35].

The most widely used technique for diagnosing *O. lupi* infections involves a combination of morphological characterization of skin-dwelling microfilariae or adult specimens by microscopy and DNA detection by conventional PCR (cPCR). Skin snip samples are preferably collected from the nose, forehead, and interscapular region [11–13, 16, 17, 20, 22–24, 39, 40]. Diagnostic imaging, real-time PCR (qPCR), and indirect ELISA techniques have been recently developed and optimized to detect *O. lupi* in animals and humans [14, 41–47]. However, these diagnostic techniques can be invasive, labor-intensive, and prone to PCR inhibition, resulting in false negatives. Droplet digital PCR (ddPCR) is an advanced molecular technique that enables absolute quantification of target DNA without the need for external standards [48, 49]. In ddPCR, the reaction mixture containing the DNA sample, primers, probes, and other components is partitioned into thousands of nanoliter-sized droplets, each acting as an independent PCR reaction [48, 49]. After amplification, each droplet is analyzed for the presence or absence of the target sequence using a fluorescent signal. By applying Poisson statistics, ddPCR calculates the absolute number of target molecules in the original sample without relying on standard curves [48, 49]. The importance of ddPCR lies in its high sensitivity and accuracy, particularly for detecting low-abundance targets that may be missed by cPCR or qPCR. It is less affected by PCR inhibitors, making it ideal for challenging samples such as host tissues or formalin-fixed specimens [48, 49]. Droplet digital PCR has been increasingly utilized in clinical diagnostics, epidemiological surveillance, and research for pathogen detection, mutation analysis, and monitoring treatment efficacy [48, 49]. Therefore, our objective was to validate a ddPCR assay for detecting *O. lupi* across various sample types, including adult *O. lupi* fragments, subconjunctival nodule biopsies, skin snips, and formalin-fixed paraffin-embedded (FFPE) tissues of both dogs and cats. Then, the performance of this novel assay was compared to cPCR and qPCR to assess the prevalence in suspected clinical cases.

Methods

Suspected clinical cases

Specimens from suspected clinical cases of *O. lupi* from both dogs (n = 196) and cats (n = 6) were submitted by veterinary ophthalmologists and pathologists from 11 states within the US, between 2018 and 2025, to the Texas A&M University Parasitology Diagnostic Lab (Fig. 1). All samples collected complied with the animal use protocol approved by Texas A&M University's (TAMU) Institutional Animal Care and Use Committee (IACUC number: 2022 – 0261). Depending on the sample preservative (i.e., 70% ethanol vs. FFPE), different extraction methods were used. Detailed demographic data were collected for most cases; some information was missing for a proportion. Whenever possible and/or requested, these results were shared with the veterinarian responsible for the case.

Sample preparation

After collecting fresh tissue samples, veterinarians were advised to ship each sample in 70% ethanol for preservation. The samples included subconjunctival nodule biopsies, skin snips from the interscapular region, ear snips, and adult specimen fragments visible during collection. Following arrival, samples were transferred from a collection tube to a 1.5 mL microcentrifuge tube to evaporate ethanol using an Eppendorf Vacufuge (Eppendorf Vacufuge® Plus, Eppendorf, Germany), thereby reducing the likelihood of inhibition

during genomic extraction. Before genomic extraction, all fresh tissue samples were enzymatically and mechanically disrupted by incubating in a thermomixer (Eppendorf, Germany) overnight at 56°C with a rotation rate of 350 RPM.

All FFPE samples received contained at least 1–2 scrolls, each 20 μM thick. Upon arrival, the samples were placed in 1.5 mL microcentrifuge tubes. Genomic DNA was extracted following the protocol described above. When multiple FFPE scrolls from a single animal were received (6 scrolls total), the scrolls were individually processed, with two scrolls used per extraction. Once completed, a compiled sample containing 5 μL from each DNA extraction was used to screen for *O. lupi*.

Genomic DNA was then extracted from all tissue samples using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Genomic DNA from all FFPE samples was extracted using the QIAmp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. All genomic DNA was then kept frozen at -20°C until molecular testing.

Molecular-based diagnostic assays

Conventional PCR

Conventional PCR was performed using pan-filarial primers targeting the mitochondrial cytochrome oxidase c subunit 1 (*cox1*), yielding an amplicon of approximately 635 base pairs (bp), as previously described (14, 50) (Table 1). Briefly, all reactions were performed in a 25 μL reaction containing 8.75 μL molecular-grade water, 0.625 μL (10 μM) of each primer, 12.5 μL 2x GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), and 2.5 μL DNA template. Cycling conditions consisted of 95°C for 2 minutes, followed by 40 cycles at 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 90 seconds, and a final extension step at 72°C for 5 minutes. All runs included DNA from a positive control and a no-template control. The positive control was DNA extracted from an adult female *Setaria equina*, and the no-template control consisted of nuclease-free water. Conventional PCR products were subjected to a 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (GoldBio, St. Louis, MO, USA). The agarose gel was visualized under UV light to detect amplicons; if detected, the amplified DNA was purified using the Cycle Pure E. Z. N. A. kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Following purification, samples were sent for Sanger sequencing (Eurofins, Louisville, KY, USA). Generated sequences were compared with genetic data available in GenBank using BLAST and aligned for phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA) X [51]. If sequencing was inconclusive, further analysis using an additional set of primers targeting a shorter *cox1* fragment (115 bp) [38]. With the identical cPCR volume, reaction conditions, and purification protocol described above, the only alteration was to the annealing step at 50°C for 45 seconds for the shorter target region [38].

Table 1
Primers and probes designed for conventional PCR, real-time PCR, and droplet digital PCR for the detection of *Onchocerca lupi* DNA targeting the cytochrome c oxidase subunit (*cox1*) gene region.

Name	Sequence (5'-3')	Reference
Conventional PCR		
COIintForward	GGAGGCGGTCCTGGTAGTAG	[50]
COIintReverse	TGATTGGTGGTTTTGGTAA	[50]
ShortCOIF	TCAAAATATGCGTTCTACTGCTGTG	[38]
ShortCOIR	CAA AGACCCAGCTAA AACAGGAAC	[38]
Real-time PCR		
TaqMan® Probe	FAM-CTTAGAGTAGAGGGTCAGCC-MGB-NFQ	[14]
O.l.Forward	GGAGGCGGTCCTGGTAGTAG	[14]
O.l.Reverse	GCAAACCCAAAACTATAGTATCC	[14]
Droplet Digital PCR		
Iowa Black Probe	FAM-CTTAGAGTA /ZEN/ GAGGGTCAGCC-IABkFQ	Current Study
O.l.Forward	GGAGGCGGTCCTGGTAGTAG	Current Study
O.l.Reverse	GCAAACCCAAAACTATAGTATCC	Current Study

Probe-based qPCR

All sample types were subjected to a recently optimized probe-based qPCR targeting a short region of *cox1*, which amplified a 90 bp fragment of *O. lupi* [14] (Table 1). Briefly, all reactions consisted of 1.5 µL molecular-grade water, 0.5 µL (50 µM) each primer, 0.5 µL (20 µM) of probe, 10 µL 2x of TaqMan® Fast Advance Master Mix (Applied Biosystems, Waltham, MA, USA), 1 µL of VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay (ThermoFisher Scientific Inc., Waltham, MA, USA), 1 µL of VetMAX™ Xeno™ Internal Positive Control ($10^{-2.5}$) (ThermoFisher Scientific Inc., Waltham, MA, USA), and 5 µL of DNA template for a 20 µL reaction volume [14]. All qPCR assays were performed on a QuantStudio 3 real-time PCR system (Applied Biosystems, Waltham, MA, USA). Following the previously published protocol [14], all skin snips were diluted 1:10. In contrast, adult specimens visible during biopsies, subconjunctival nodule biopsies, ear snips, and FFPE samples were undiluted when tested [14]. Cycling conditions included an initial denaturation step at 95°C for 3 minutes to allow DNA separation, followed by 40 cycles of a two-step PCR at 95°C for 10 seconds and 64°C for 30 seconds. All runs included two positive controls and a non-template control. One positive control included extracted DNA from an adult *O. lupi* specimen, confirmed morphologically and molecularly. The second positive control consisted of the diluted DNA from VetMAX™ Xeno™ ($10^{-2.5}$). For the no-template control, nuclease-free water was used. Results of qPCR were analyzed using Design & Analysis 2 software to determine the cycling threshold (CT) value of each sample (Applied Biosystems, Waltham, MA, USA) [14].

Droplet digital PCR

Optimization of droplet digital PCR protocol

The probe and primers used in this ddPCR were originally developed for a probe-based qPCR targeting mitochondrial *cox1* to detect the single genetic lineage of *O. lupi* reported in the US [14]. In the present study, we adapted the original probe to an Iowa Black Probe® (Integrated DNA Technologies, Coralville, IA, USA) labeled with a FAM reporter dye (Table 1). First, we evaluated the optimal concentration for ddPCR using a ten-fold serial dilution series (10^{-1} to 10^{-5}) with four sample types: i) an *O. lupi* adult fragment DNA, ii) a subconjunctival nodule biopsy, iii) a skin snip from the interscapular region, and iv) an FFPE of a subconjunctival nodule biopsy, all of which were positive in both cPCR and qPCR.

To determine the optimal annealing temperature, we then performed a temperature gradient from 56.0°C to 66.0°C to assess droplet separation using three of the commonly received sample types: an adult *O. lupi* specimen and a subconjunctival nodule, both at a 10^{-1} dilution, and an undiluted FFPE of a subconjunctival biopsy. To evaluate the assay's ability to detect low-copy number targets, a serial dilution was prepared using synthetic DNA corresponding to the *O. lupi cox1* gene region (molecular weight: 67,435.6 g/mol). The synthetic DNA had an initial concentration of 20 ng/μL (ThermoFisher Scientific Inc., Waltham, Massachusetts, USA). A working solution for the ddPCR with 1 x 10^7 copies/μL was prepared by diluting a 0.2 ng/μL stock with nuclease-free water. From this working solution, a tenfold serial dilution was performed to yield concentrations ranging from 10^6 to 10^0 copies/μL in the total reaction volume (20 μL).

Given the high analytical sensitivity of ddPCR, we evaluated the potential cross-reactivity. All sample types assessed in this study, including adult specimen fragments, subconjunctival nodule biopsies, skin snips, ear snips, and FFPE tissues, were included in the analysis. Samples used to assess cross-reactivity were considered "negative control samples" and included an adult *D. immitis* specimen, tissue DNA from a purpose-bred laboratory beagle, and an FFPE tissue sample from a bat, all of which were *O. lupi*-negative.

All reactions consisted of 5.5 μL molecular-grade water, 1.1 μL (9 μM) each primer, 1.1 μL (5 μM) of probe, 11 μL of ddPCR™ Supermix for Probes (no dUTP) (Bio-Rad Laboratories Inc., Hercules, CA, USA), and 2.2 μL of DNA template for a 22.2 μL reaction volume. Reactions were loaded into 96-well cartridges, and droplets were generated using the QX200™ AutoDG Droplet Generator (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR amplification was carried out on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using manufacturer recommended cycling conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, and an annealing/extension step of 60°C for 1 min, with a final enzyme deactivation step at 98°C for 10 minutes. Nuclease-free water was used as a no-template control throughout all assays. Once amplified, samples were analyzed using the QX200 Droplet Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All droplet fluorescence data were analyzed using QX Manager Software, 2.2 Standard Edition (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Testing clinical samples

Using the newly optimized ddPCR assay targeting a 90 bp fragment of the *cox1* gene of *O. lupi* (Table 1), all sample types were screened. All reactions consisted of the same reagents as described above. Both adult specimen fragments and subconjunctival nodules samples were diluted 10^{-1} for optimal droplet separation, whereas the remaining sample types were undiluted when tested. Positive controls included DNA of an adult *O. lupi* specimen, a subconjunctival biopsy, and an FFPE tissue sample, all confirmed morphologically and molecularly. Negative controls were those that tested during cross-reactivity (i.e., an adult specimen of *D. immitis*, tissue from a purpose-bred laboratory Beagle, and an FFPE tissue sample from a bat) that were confirmed *O. lupi*-negative. The no-template control was nuclease-free water.

Statistical Analysis

Statistical analyses were conducted using STATA®, version 19.5 BE-Basic Edition (College Station, TX, USA). All descriptive statistics and positivity reported across each diagnostic technique are summarized below. We performed Cohen's Kappa (κ) to determine the level of agreement between each paired diagnostic test [52–54]. The level of agreement was categorized as follows: $\kappa \leq 0$, no agreement; $\kappa = 0.01–0.2$, slight agreement; $\kappa = 0.21–0.4$, fair agreement; $\kappa = 0.41–0.6$, moderate agreement; $\kappa = 0.61–0.8$, substantial agreement; $\kappa = 0.81$-1.0, almost perfect [52–54]. We also applied the Cochran's Q test to assess the binary results across all three diagnostic tests. A post hoc analysis using McNemar's test was performed, with each diagnostic test paired, and controlled for multiple comparisons with the incorporation of the Bonferroni correction. A *p*-value of ≤ 0.05 was considered statistically significant unless adjusted [52].

Results

ddPCR optimization and validation

The adult fragment and the subconjunctival nodule biopsy showed sufficient rain (Fig. 2). Undiluted samples did not show two distinct clusters, indicating the DNA concentration used in the reaction was very high, whereas the 10x dilution showed two distinct clusters of

positive and negative droplets (Fig. 2A and 2B). Therefore, a dilution factor of 10^{-1} was applied for all adult specimens and conjunctiva nodules samples. For the remaining sample types, including skin snips, ear snips, and FFPE samples, no dilution factor was required due to ideal droplet separation (Fig. 2C and 2D. The temperature gradient across all three sample types tested (i.e., a 10^{-1} dilution of an adult specimen, a 10^{-1} dilution of a subconjunctival nodule biopsy, and undiluted FFPE of a subconjunctival nodule biopsy) showed ideal droplet separation following the manufacturer's conditions (60°C); therefore, universal cycling conditions were implemented (Fig. 3). When assessing target DNA concentration using synthetic DNA, positive droplets were observed at all concentrations, with the lowest concentration (10^{0}) yielding a single positive droplet, indicating that the assay is capable of detecting extremely low copy numbers approaching the single-molecule level under universal cycling conditions (Fig. 4). Evaluation of cross-reactivity revealed that all *O. lupi*-negative samples produced exclusively negative droplets, confirming the assay's analytical specificity (Fig. 5).

Suspected clinical cases

A total of 202 suspected clinical cases were screened, of which 97.0% (n = 196/202) originated from dogs and 3.0% (n = 6/202) from cats. Five different tissue types were evaluated, including subconjunctival nodule biopsies (n = 103/202), adult specimen fragments (n = 34/202), skin snips (n = 23/202), ear snips (n = 3/202), and FFPE tissue samples (n = 39/202) (Table 2). Diagnostic outcomes for each molecular assay are summarized in Table 3. Overall, ddPCR showed the highest number of positive samples (71.3%, n = 144/202; 95% CI: 64.5—77.4), followed by qPCR (64.3%, n = 130/202; 95% CI: 57.3—70.9), with the fewest by cPCR and sequencing (54.0%, n = 109/202; 95% CI: 46.8—60.9) (Table 3). In the ddPCR, we confirmed 100% (n = 34) of adult specimen fragments and 58.2% (n = 60/103) of subconjunctival samples as positive at a 10^{-1} dilution factor (Table 3). Notably, ddPCR also identified seven additional positive skin snips (n = 13/23) that were not detected by either cPCR or qPCR. Using this optimized assay, we detected *O. lupi* in 94.9% (n = 37/39) of the FFPE samples (Table 3).

Table 2
All tissue samples from suspected *Onchocerca lupi* clinical cases included in this study.

Sample type	**No. (%)**	**No. Dogs (%)**	**No. Cats (%)**
Subconjunctival nodule biopsies	103 (63.1)	98 (95.1)	5 (4.9)
Adult specimen fragments	34 (20.9)	33 (97.0)	1 (2.9)
Skin snip	23 (14.1)	23 (100.0)	-
Ear snip	3 (1.8)	3 (100.0)	-
Formalin-fixed paraffin-embedded tissue	39 (19.3)	39 (100.0)	-
Total	202	196	6

Table 3
Summary of results obtained using conventional PCR, real-time PCR, and droplet digital PCR for the detection of *Onchocerca lupi* DNA.

Diagnostic test(s)	**No. positive (%)**	**95% CI**
Conventional PCR (cPCR)	109 (54.0)	46.8—60.9
Subconjunctival nodule biopsies	53 (51.5)	41.4—61.4
Adult Specimen Fragments	32 (94.1)	80.3—99.2
Skin Snips	6 (26.1)	10.2—48.4
Ear Snip	0	-
FFPE tissue	18 (46.2)	30.0—62.8
Real-time PCR (qPCR)	130 (64.3)	57.3—70.9
Subconjunctival nodule biopsies	54 (52.4)	42.3—62.3
Adult Specimen Fragments	33 (97.1)	84.6—99.9
Skin Snips	8 (34.8)	16.3—57.2
Ear Snip	0	-
FFPE tissue	35 (89.8)	75.7—97.1
Droplet Digital PCR (ddPCR)	144 (71.3)	64.5—77.4
Subconjunctival nodule biopsies	60 (58.2)	48.1—67.8
Adult Specimen Fragments	34 (100.0)	89.7—1.00
Skin Snips	13 (56.5)	34.4—76.8
Ear Snip	0	-
FFPE tissue	37 (94.9)	82.6—99.3
Total	202	
(FFPE: formalin-fixed paraffin-embedded; CI: confidence interval)		

Using the probe-based qPCR, 64.3% (n = 130/202) were positive for *O. lupi*, of which an additional 21 positive samples for *O. lupi* were obtained when compared to cPCR, including one adult fragment, one subconjunctival biopsy, nineteen FFPE tissue samples, and two skin snips (Table 3). Conventional PCR confirmed 54.0% (n = 109/202), yielding 96 conclusive sequences with the pan-filarial *cox1* primers [50] and 13 conclusive sequences via the shorter *cox1* target [38] (Table 3). However, two of the sequences obtained and identified (from FFPE tissue samples) were previously published using both primer sets [23, 24]. Therefore, a total of 107 sequences were obtained within this study. Using the pan-filarial primers, all 95 identical sequences obtained [50] were deposited in GenBank (Accession Number: PX857514-608) and were 100% identical to *O. lupi* sequences from the USA. The remaining 12 samples amplified identical sequences with the shorter *cox1* target previously published [38], but were too short in bp to be submitted to GenBank. Therefore, we have included all 12 sequences in Supplementary Data, File 1. Additionally, we have listed all available information for each conclusive sequence submitted to GenBank (i.e., accession number, geographical location, and sample type) (Supplementary Data, Table S1) as well as those sequences via the short *cox1* target [38] (i.e., geographical location, and sample type) (Supplementary Data, Table S2). We did not detect any *O. lupi* DNA within the three ear skin snips screened across all three diagnostic tests. Overall, 71.9% dogs (n = 141/196) and 50.0% cats (n = 3/6) were positive via this ddPCR assay (Table 4). Regarding confirmed canine *O. lupi* infections, these originated from the majority of states from which samples were submitted (n = 10/11), with the largest number of positive samples from New Mexico (70.3%; n = 97/138). This was also observed in cats from New Mexico with the most positive results (40.0%; n = 2/5). There were no positive dogs from Tennessee (n = 0/5).

Table 4
Summary of all *Onchocerca lupi* positive clinical cases by sampling location.

Collection States	Total per state			Positive via cPCR			Positive via qPCR			Positive via ddPCR		
	No.	Dogs	Cats	No. (%)	Dogs (%)	Cats (%)	No. (%)	Dogs (%)	Cats (%)	No. (%)	Dogs (%)	Cats (%)
Arizona	10	10	-	5 (50.0)	5 (50.0)	-	8 (80.0)	8 (80.0)	-	9 (90.0)	9 (90.0)	-
California	10	10	-	5 (50.0)	5 (50.0)	-	10 (100.0)	10 (100.0)	-	10 (100.0)	10 (100.0)	-
Colorado	11	10	1	6 (54.5)	5 (50.0)	1 (100.0)	9 (81.8)	8 (80.0)	1 (100.0)	9 (81.8)	8 (80.0)	1 (100.0)
Georgia*	1	1	-	1 (100.0)	1 (100.0)	-	1 (100.0)	1 (100.0)	-	1 (100.0)	1 (100.0)	-
Illinois*	1	1	-	1 (100.0)	1 (100.0)	-	1 (100.0)	1 (100.0)	-	1 (100.0)	1 (100.0)	-
New Mexico	143	138	5	85 (59.4)	83 (55.8)	2 (40.0)	87 (61.0)	85 (61.6)	2 (40.0)	99 (69.2)	97 (70.3)	2 (40.0)
North Carolina*	5	5	-	1 (20.0)	1 (20.0)	-	1 (20.0)	1 (20.0)	-	1 (20.0)	1 (20.0)	-
Oklahoma*	2	2	-	0	-	-	2 (100.0)	2 (100.0)	-	2 (100.0)	2 (100.0)	-
Tennessee	5	5	-	0	0	-	0	0	-	0	0	-
Texas	5	5	-	2 (40.0)	2 (40.0)	-	3 (60.0)	3 (60.0)	-	3 (60.0)	3 (60.0)	-
Utah	9	9	-	3 (33.3)	3 (33.3)	-	8 (88.9)	8 (88.9)	-	9 (100.0)	9 (100.0)	-
Total	202	196	6	109 (54.0)	106 (54.1)	3 (50.0)	130 (64.4)	127 (64.8)	3 (50.0)	144 (71.3)	141 (71.9)	3 (50.0)

(*First confirmed case; cPCR: conventional PCR; qPCR: real-time PCR; ddPCR: droplet digital PCR)

As shown in Table 5, Cohen's Kappa showed moderate agreement between cPCR + qPCR (κ = 0.74), with almost perfect agreement between qPCR + ddPCR (κ = 0.81), and fair agreement was found between cPCR + ddPCR (κ = 0.60). Cochran's Q revealed a significant difference among the three diagnostic methods ($p < 0.001$) (Table 6). In the post hoc analysis, differences remained significant across all three diagnostic tests after Bonferroni correction (Table 7).

Table 5
The level of agreement found between each molecular diagnostic test used for the detection of *Onchocerca lupi*.

Comparison of two tests (Test 1/Test 2)	a	b	c	d	Cohen's Kappa (κ)	Agreement Interpretation
cPCR/qPCR	107	2	23	70	0.7467	Moderate
cPCR/ddPCR	107	2	37	56	0.6004	Fair
qPCR/ddPCR	129	1	15	57	0.8195	Almost Perfect

(cPCR: conventional PCR; qPCR: real-time PCR; ddPCR: droplet digital PCR)

Table 6
Cochran's Q test results for comparing three molecular diagnostic tests individually to detect *Onchocerca lupi*.

Comparison of individual methods	**Q statistic** **df** ***p*-value**
cPCR	46.5500
qPCR	2
ddPCR	**< 0.001**

(Significant relationships ($p < 0.05$) are denoted by bold font. df: degrees of freedom; cPCR: Conventional PCR; qPCR: probe-based real-time PCR; ddPCR: droplet digital PCR;)

Table 7
Post-hoc analysis of the Cochran's Q test for individual molecular tests using a McNemar test.

Post hoc analysis	***p*-value**
cPCR/qPCR	**< 0.001***
cPCR/ddPCR	**< 0.001***
qPCR/ddPCR	**< 0.001***

(*Significant after Bonferroni correction (=0.0167); cPCR: Conventional PCR; qPCR: probe-based real-time PCR; ddPCR: droplet digital PCR;)

Discussion

In this study, we developed and optimized a highly sensitive and specific probe-based ddPCR assay capable of detecting *O. lupi* DNA at very low copy numbers. We also assessed the most extensive collection of suspected clinical cases of *O. lupi* in the US, comprising cases received from 11 states. Of these, four states (i.e., Georgia (n = 1/1), Illinois (n = 1/1), North Carolina (n = 1/5), Oklahoma (n = 2/2)) had their first confirmed *O. lupi* clinical case (Table 4). However, due to the absence of a complete history for these dogs, we cannot affirm whether these cases were autochthonous or travel-related. Among the various sample types received, we evaluated *O. lupi* infection across five tissue types: adult specimen fragments, subconjunctival nodule biopsies, skin snips, ear snips, and FFPE tissue. With the high sensitivity of ddPCR, we were able to detect DNA from an adult specimen stored in formalin for several years, which was negative in cPCR and qPCR.

Among the collected samples, the majority were from dogs (n = 196). Among the cat samples, we received six suspected cases, of which 50% (n = 3/6) were confirmed positive for *O. lupi* by all three diagnostic techniques. Of the positive cat cases, one was an adult specimen fragment, and the other two were subconjunctival samples. Two of the three cat samples originated in New Mexico, and the third in Colorado. From the two previously reported cat cases in the US, the first originated in Utah, with a travel history from southern Nevada, and the second was permanently housed in a shelter in southern Utah [20]. The only large-scale epidemiological study performed was in Portugal, which sampled 155 stray cats via ear-snipping and identified a single cat infected with *O. lupi* [27]. The most recent case of *O. lupi* outside the US was in a client-owned cat from Romania with no travel history and free-range outdoor access [28]. Additionally, a recent epidemiological study is the only other study assessing *O. lupi* prevalence in cats in the US and reports an overall prevalence of 9.4% in cats and dogs. However, it is unclear what proportion of cats tested positive for *O. lupi*, separating species prevalence not the study's main objective [15]. Interestingly, all four reported cat cases worldwide, as well as the three identified in the present study, originated from regions where *O. lupi* has been documented in dogs, indicating that cats may play a secondary role for *O. lupi* in the epidemiology of this parasite compared to dogs [10, 18, 19, 21, 25, 40]. These findings also suggest that cats may serve as reservoirs in endemic areas, thereby potentially contributing to vector transmission dynamics [15, 38]. Given the limited information on the clinical and epidemiological aspects of *O. lupi*, further studies are needed to assess baseline prevalence at local-, national-, and global-scale.

This newly developed ddPCR assay was adapted from a recently published probe-based qPCR assay in our lab, which was implemented in the largest epidemiological study of shelter dogs in an endemic urban area within the southwestern US [14]. Following

optimization, the original qPCR showed no cross-reactivity with other filarial nematodes found in blood (i.e., *Dirofilaria immitis, Acanthocheilonema reconditum*) and skin (i.e., *Cercopithifilaria bainae*) [14, 37], all of which are known to co-occur with *O. lupi* in the US. This result was consistent with the ddPCR assay, as we found no cross-reactivity among the three *O. lupi*-negative sample types, including one containing DNA of an adult *D. immitis* specimen. The high diagnostic sensitivity and specificity observed with ddPCR indicate a low likelihood of false positives, unlike with other molecular techniques, such as qPCR and cPCR [14]. Given the host-agnostic capability of this novel ddPCR, its high sensitivity and specificity, and the limited availability of diagnostic techniques, such as histopathology, imaging, and qPCR, it can also be easily implemented to confirm human cases [29, 35, 44].

In this opportunistic study, 10 of 11 sampled states had at least one confirmed *O. lupi* case via ddPCR (Table 4). Over half of the states from which samples were submitted are in the southwestern US (n = 6/11), where *O. lupi* is known to be endemic [11–15]. The remaining positive cases were from four states outside the southwestern US, where *O. lupi* endemicity had not previously been confirmed (Table 4) [22, 33, 55]. One major limitation of this study was that the sampled companion animals did not report a state of origin, state of adoption, or travel history (recent or former), which could not be provided for every suspected clinical case. Although veterinary ophthalmologists and pathologists submitted these samples, owners may not have shared this information at the time of sampling or, more likely, may not have known it. Additionally, some cases may have remained subclinical for many years and only showed clinical signs at the time of sampling, highlighting the importance of recording the origin of adoption in clinical assessments. Although it is not readily available due to a lack of records from adoption through a breeder or shelter, travel history must be recorded by attending veterinarians, as *O. lupi* continues to emerge and may be reported from areas of unknown endemic status [11–13, 22, 55].

For *O. lupi*, little is known about whether specific biological factors, such as age, sex, breed, and coat color, increase the risk of infection. A limitation of this study was that age was not available for all clinical cases. However, among those reported, the youngest age for an *O. lupi*-positive dog from a subconjunctival sample was 1 year and 7 months. In addition, a recent study from our lab found that two of eight shelter dogs positive for *O. lupi* were juveniles (≤ 1 year old) [14]. These findings suggest that the pre-patent period of *O. lupi* may be shorter than previously reported at 3–8 years in other clinical cases [3, 9, 38]. In another study, researchers assessed the relationship between coat color and black flies (*Simulium* spp.) in dogs in New Mexico, finding that larger dogs with black coats had a higher risk of *O. lupi* infection than smaller dogs [16]. Biological factors such as age and coat color need to be assessed in controlled experimental infections, and future active surveillance studies of companion animals are critical for fully elucidating the biology and epidemiology of *O. lupi* across its geographic distribution.

Although initially introduced in the 1990s, ddPCR remains relatively new in DNA-based techniques in infectious disease and parasitology diagnostics, especially in the veterinary field, where its capacity for absolute quantification represents a transformative advancement for accurate pathogen detection [48, 49, 55, 57]. In veterinary parasitology, ddPCR has recently been implemented to assess signaling pathways, detect drug resistance, and improve current molecular diagnostics [58–62]. However, ddPCR has limitations in terms of cost and clinical diagnostic utility [48, 49, 56]. One major limitation of ddPCR is the higher cost of instruments and reagents, which makes its use more difficult, even in reference laboratories, as it is not widely available [48, 49, 56]. This is essential for identifying current or past infections to aid in treatment protocols, which is why incorporating a combination of classical parasitology using microscopy-based techniques and highly accurate molecular-based tools is essential for reliable diagnosis [11, 14, 22, 23].

Conclusion

This novel ddPCR assay demonstrated high sensitivity and specificity for detecting *O. lupi* across five sample types of suspected companion animal clinical cases, providing greater accuracy than cPCR and qPCR. This study also represents the most extensive series of confirmed clinical cases in the US, with 10 of 11 states sampled having at least one *O. lupi* positive case, and four states with the first confirmed clinical case. These results suggest that susceptible hosts face an increased risk of infection in both endemic and non-endemic areas. In clinical settings, this ddPCR assay can serve as a standard diagnostic test in reference laboratories to accurately evaluate suspected cases and can be used in large-scale surveillance studies to identify subclinical infections in various populations, such as shelter and stray dogs. Using molecular diagnostics, such as ddPCR, can elucidate the biology and epidemiology of this zoonotic filarial nematode and support control and prevention efforts.

Declarations

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Author's contributions

MAK: Technical writing – original draft, data curation, laboratory optimization and diagnostic work, methodology validation, formal analysis, statistical analysis. **NJM:** Data curation and technical writing—review and editing. **SP:** Data curation and technical writing—review and editing. **SF:** Data curation and technical writing—review and editing. **TY:** Data curation and technical writing—review and editing. **BF:** Data curation and technical writing—review and editing. **JC:** Data curation and technical writing—review and editing. **Jordan West:** Data curation and technical writing—review and editing. **KB:** Data curation and technical writing—review and editing. **CS:** Supervision, laboratory optimization, data curation, and technical writing—review and editing. **HH:** Supervision, laboratory optimization and diagnostics work, methodology validation, technical writing – review and editing. **PW:** Supervision, laboratory optimization and diagnostics work, methodology validation, technical writing – review and editing. **GGV:** Supervision, conceptualization, visualization, resources, funding, technical writing – review and editing.

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Data availability

Data available upon request.

Ethics approval and consent to participate

Collection of samples followed the protocols approved by Texas A&M University's Institutional Animal Care and Use Committee under protocol number 2022-0261.

Consent for publication

All authors consent to the publication of this manuscript.

Competing interests

The authors declare no competing interests.

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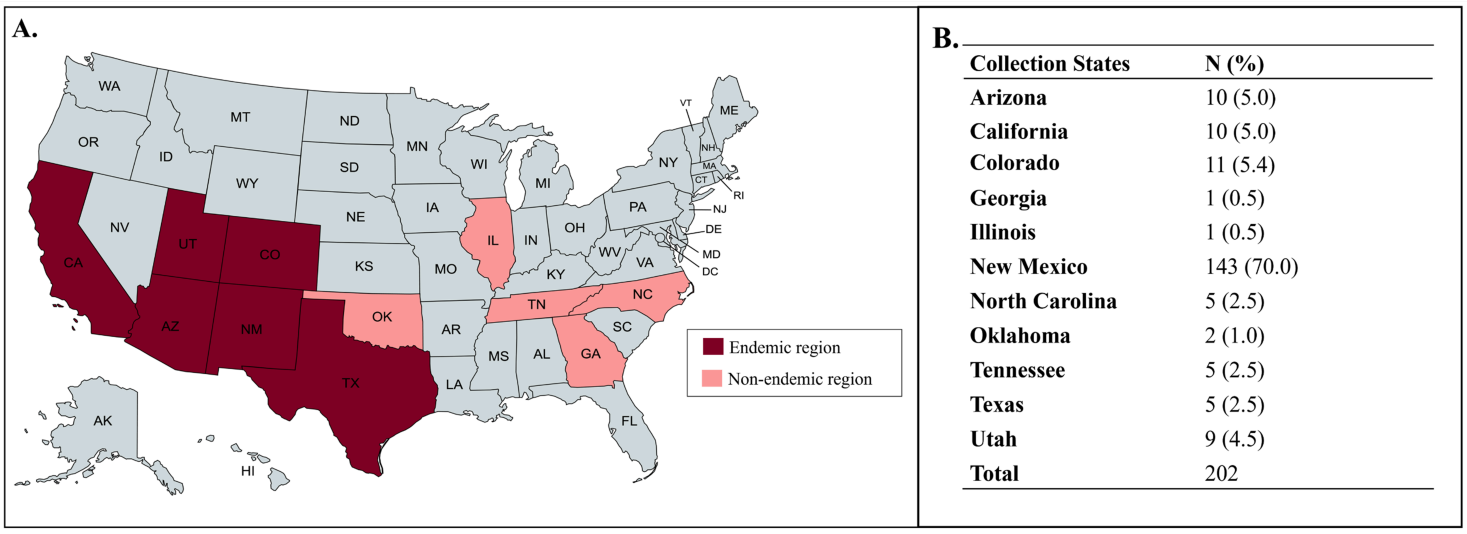
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Figures



B.

Collection States	N (%)
Arizona	10 (5.0)
California	10 (5.0)
Colorado	11 (5.4)
Georgia	1 (0.5)
Illinois	1 (0.5)
New Mexico	143 (70.0)
North Carolina	5 (2.5)
Oklahoma	2 (1.0)
Tennessee	5 (2.5)
Texas	5 (2.5)
Utah	9 (4.5)
Total	202

Figure 1

A map showing all 11 states sampled from, highlighting the endemic and non-endemic regions (A), with a summary of all suspected clinical cases per state (B).

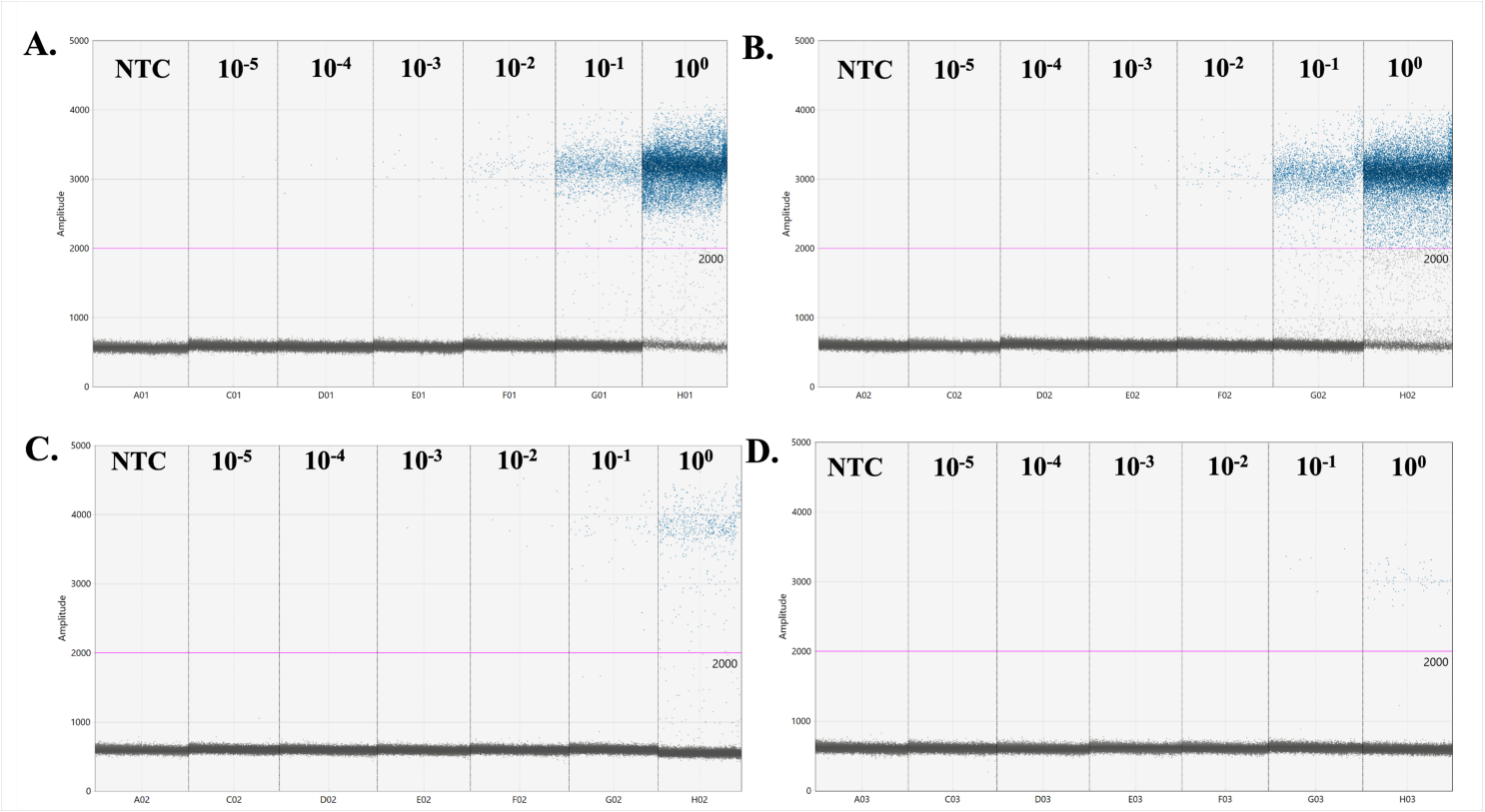


Figure 2

A visual representation of ddPCR droplets generated from a tenfold dilution (1 to 10^{-5}) test using DNA of four different sample types: (A) an adult *Onchocerca lupi* specimen, (B) a subconjunctival nodule biopsy, (C) a skin snip, and (D) a formalin-fixed paraffin-embedded subconjunctival tissue sample. The minimum threshold for positive droplets is indicated by the pink link, with positive droplets (blue) and negative droplets (grey).

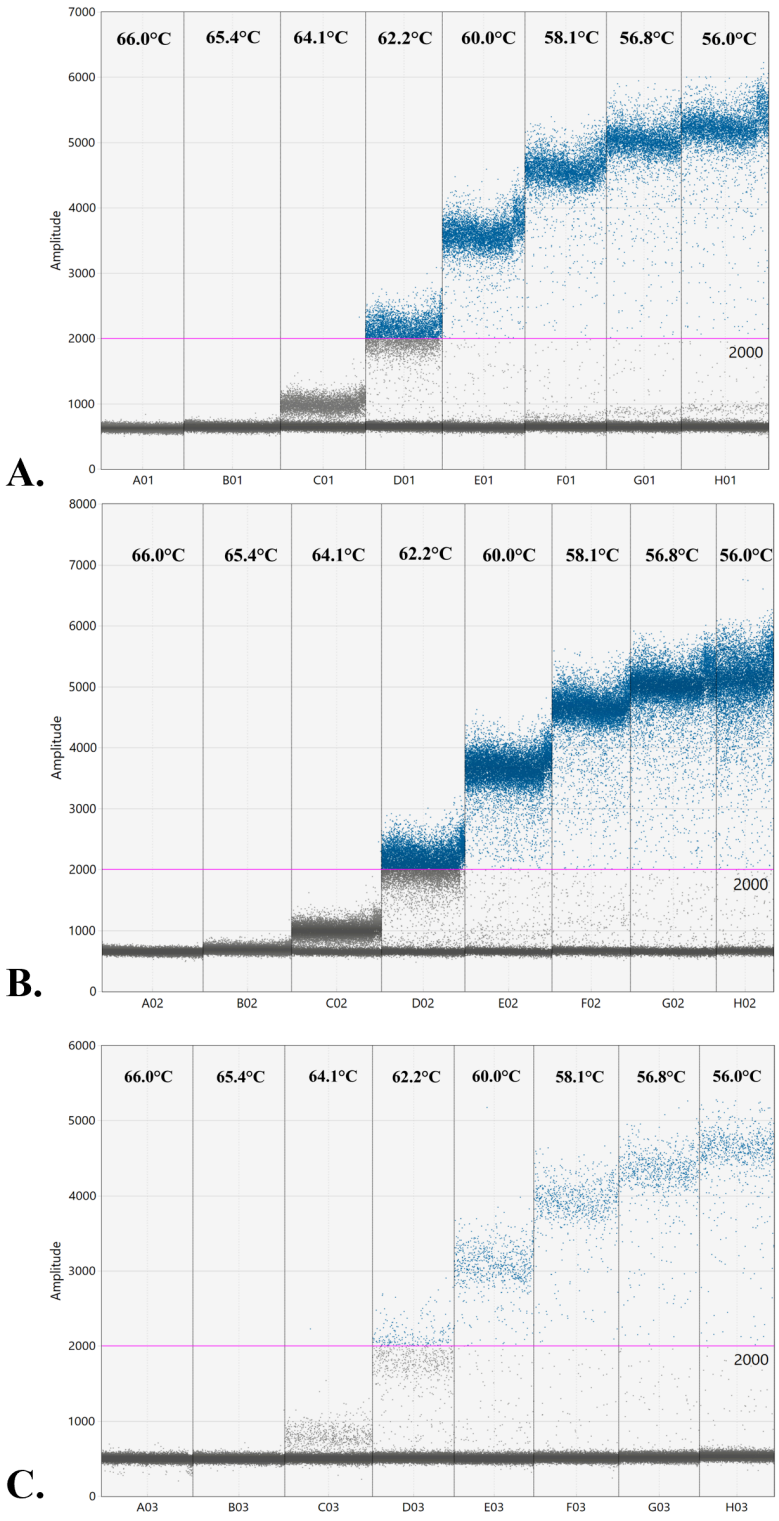


Figure 3

A visual representation of ddPCR droplets generated during temperature gradient (56.0°C to 66.0°C) for droplet separation testing using three different sample types: (A) an adult *Onchocerca lupi* specimen (10^{-1}), (B) a subconjunctival nodule biopsy (10^{-1}), and (C) a formalin-fixed paraffin-embedded subconjunctival tissue sample (10^{0}).

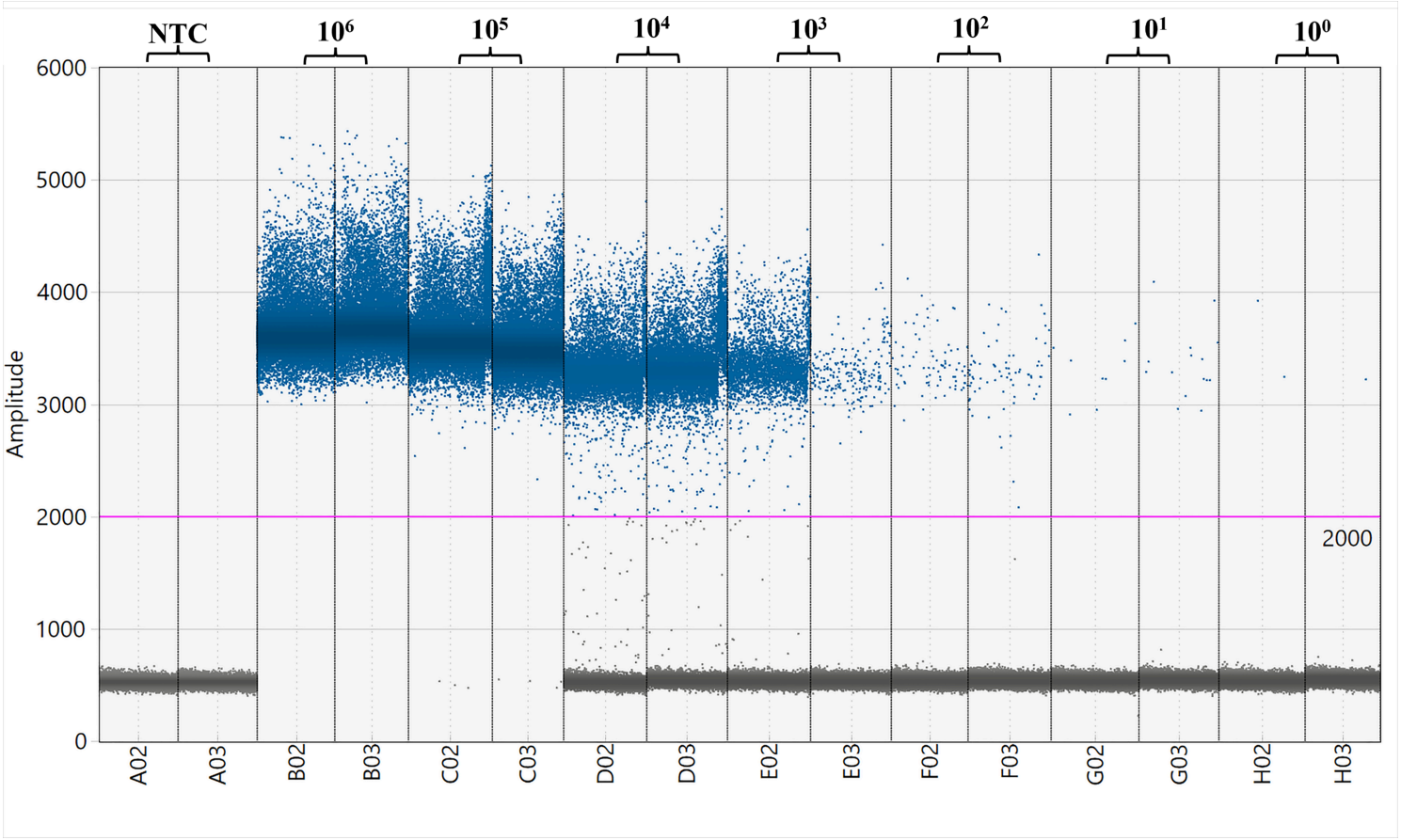


Figure 4

A visual representation of ddPCR droplets generated to assess a single copy of the target DNA using synthetic DNA derived from an adult *Onchocerca lupi* specimen. The minimum threshold for positive droplets is indicated by the pink link, with positive droplets (blue) and negative droplets (grey) shown at a serial dilution (10^6 to 1).

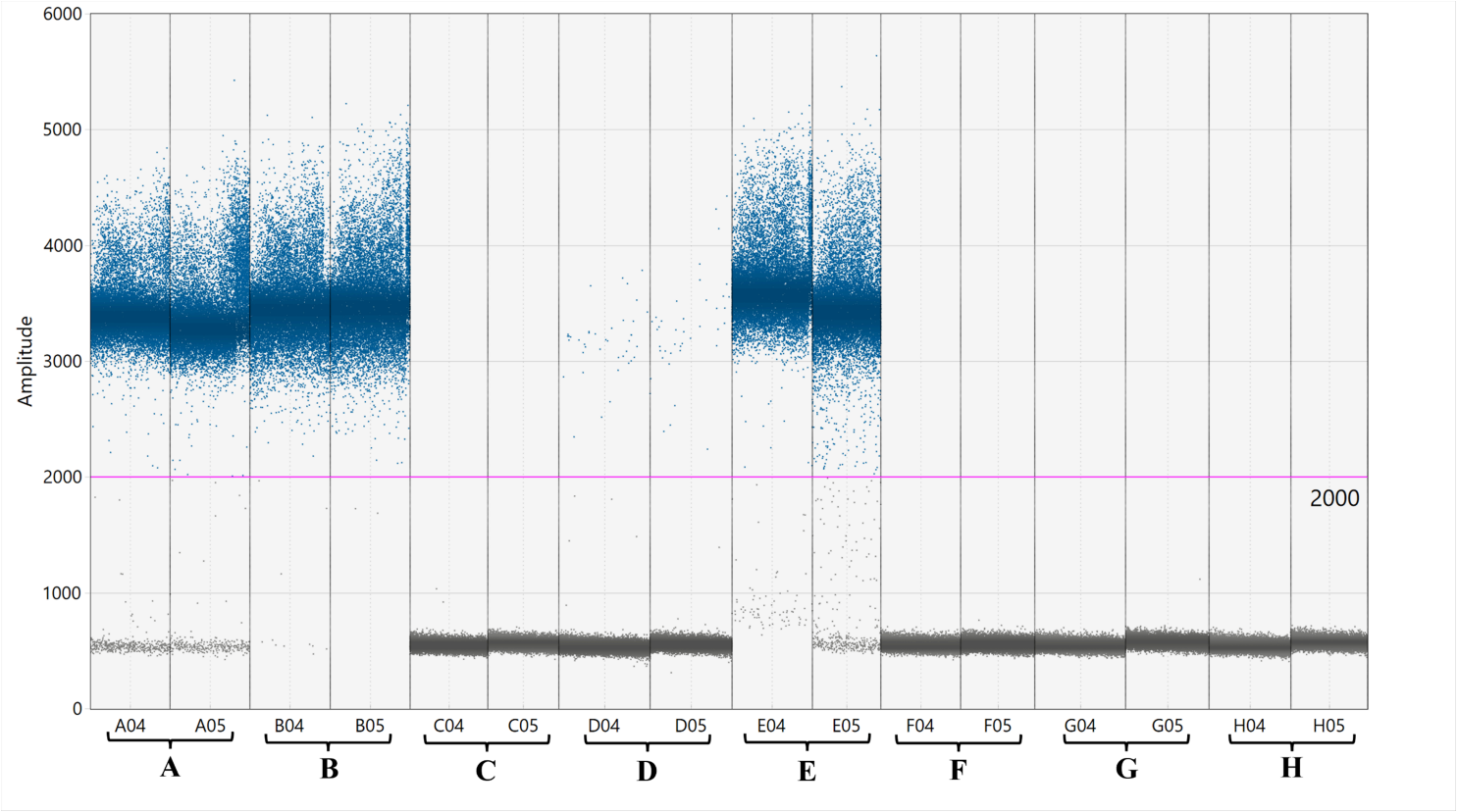


Figure 5

A visual representation of ddPCR droplets generated from using DNA from five suspected tissue samples, including (A) an adult *Onchocerca lupi* specimen, (B) a subconjunctival nodule biopsy, (C) a skin snip, (D) an ear snip, and (E) a formalin-fixed paraffin-embedded subconjunctival tissue sample. Negative controls included (F) an adult *Dirofilaria immitis* specimen, (G) a skin snip from a purpose-bred laboratory canine, and (H) a formalin-fixed paraffin-embedded tissue sample from a bat. The minimum threshold for positive droplets is indicated by the pink link, with positive droplets (blue) and negative droplets (grey) shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OLddPCRSupplementaryTables41326.docx
- OLddPCRSupplementaryDataFile14726.txt