

Cross-Sectional Study of Soil-Transmitted Helminthiases in Black Belt Region of Alabama, USA

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We conducted a cross-sectional study to determine the prevalence of soil-transmitted helminthiases (STH) in areas of rural Alabama, USA, that have sanitation deficits. We enrolled 777 children; 704 submitted stool specimens and 227 a dried blood spot sample. We microscopically examined stool specimens from all 704 children by using Mini-FLOTAC for helminth eggs. We tested a subset by using molecular techniques: real-time PCR analysis for 5 STH species, TaqMan Array Cards for enteric helminths, and digital PCR for *Necator americanus* hookworm. We analyzed dried blood spots for *Strongyloides stercoralis* and *Toxocara* spp. roundworms by using serologic testing. Despite 12% of our cohort reporting living in homes that directly discharge untreated domestic wastewater, stool testing for STH was negative; however, 5% of dried blood spots were positive for *Toxocara* spp. roundworms. Survey data suggests substantial numbers of children in this region may be exposed to raw sewage, which is itself a major public health concern.

Safe water and sanitation are fundamental to public health (1,2). Breakdowns in those systems lead to disease and, in temperate and tropical climates, soil-transmitted helminthiases (STH). STH are parasitic infections adversely affecting health, particularly in children and pregnant women, by contributing to anemia and malnutrition (3). An estimated 1 billion persons are infected with STHs worldwide, largely in low- and middle-income countries (3). Although the wastewater infrastructure need in such countries is well-reported, underserved communities in the United States may also lack basic services,

including effective sanitation (4–6). In 2011, the United Nations' special rapporteur on the human right to safe drinking water and sanitation reported many failures in the United States (7), including in Alabama's Black Belt region (8), where many households lack effective wastewater systems (9,10). The Black Belt region, named for its rich black soils, is characterized by extreme poverty, poor health outcomes, limited healthcare access, limited economic opportunities, and other challenges (11,12).

Necator americanus hookworms were prevalent in Alabama until the mid-1900s (13). In 1929, the highest prevalence (26%–75%) was observed in coastal counties with sandy soils, whereas counties in northern Alabama had much lower prevalence (1%–5%). A moderate prevalence was noted in the Black Belt counties (Lowndes [24%], Wilcox [44%], and Perry [45%]) (14). Large-scale public health efforts, supported by the Rockefeller Sanitary Commission and the state and local health departments, attempted to eradicate hookworm. A survey of 13 Alabama counties in 1937, and again in 1951, showed a decrease in prevalence from 37% to 17% among school age children (15). However, few systematically collected data have been available since the 1950s.

In the early 1990s, rural healthcare providers in the Alabama Black Belt continued to empirically treat children for STH, but microscopically confirmed cases of hookworm were not reported (16). Subsequently, hookworm in Alabama received limited attention until a study published in 2017 reported an analysis of 55 stool samples in which 19 (35%) were positive by qPCR for *N. americanus* hookworms and 4 (7.2%) were positive for *Strongyloides stercoralis* roundworms, from a cohort of mostly adults living with poor sanitation conditions in Lowndes County (17). Those results, combined with reports of widespread wastewater sanitation failures, raised the possibility of continued STH transmission in the southeastern United States. Our study objective was

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to estimate the prevalence of STH among children in rural Alabama.

Methods

Study Design and Setting

We calculated sample size by using a prevalence range of 3%–30% on the basis of recent published reports (16,17). By using an estimated prevalence of 3% (the theoretically lowest prevalence to support ongoing transmission) (18,19) with an infinite population size and a precision of 1.5%, we determined that a sample size of 497 was needed, giving 95% binomial exact CIs of 1.7%–4.9% with 15 observed events.

We selected 3 counties in the Alabama Black Belt as the study site because of previously reported STHs in Lowndes County (17), community concerns regarding water and sanitation in Wilcox County (20), and the longstanding failure of the sewer treatment facility in Perry County (21). We invited residents of those counties to enroll their children through several recruitment strategies, including word-of-mouth by trusted community leaders, flyer distribution, and advertisements in local newspapers, social media, and radio. Any child 2–18 years of age who had resided for ≥ 1 year within the study region were eligible to enroll; however, we used community partners to help identify households most at risk on the basis of levels of poverty, known housing clusters without functioning sanitation, or living close to the failing sewer facility. Enrollment occurred during December 2019–August 2022.

Survey

We obtained informed consent from guardians and assent of children ≥ 7 years of age and administered a short paper survey. We collected demographic data, contact information, and preference for treatment by the project physician or personal physician (if an infection was found). The survey asked about possible risk factors for infections, including household sanitation type, home sewage contamination, domestic animal exposure, well-water consumption, home-grown produce consumption, international travel history, and exposure-limiting behaviors such as screen time. The survey also assessed prior treatment for STH.

Sample Collection

During December 12, 2019–March 31, 2020, we obtained finger-prick blood samples on dried blood spot cards (PerkinElmer, <https://www.perkinelmer.com>) that were shipped to the Centers for Disease Control and Prevention (CDC), Center for Global Health,

Division of Parasitic Diseases and Malaria, for multiplex serologic antibody detection for *Strongyloides stercoralis* and *Toxocara* spp. (22). We gave families at-home stool collection kits and asked them to deliver self-collected stool specimens for shipment to the University of North Carolina Chapel Hill (UNC) for analysis. During April 1, 2020–August 10, 2022, because of the COVID-19 pandemic, we stopped collecting finger-prick blood samples and asked participants to mail the self-collected stool specimens directly to the UNC laboratory in prepaid packaging. We asked participants to collect stools from 3 separate bowel movements on separate days, then fill two 50-mL collection tubes each with 15 g of stool (1 containing 15 mL of 10% formalin and another containing 15 mL of zinc polyvinyl alcohol [Zn-PVA] [Parapak; Meridian Bioscience, <https://www.meridianbioscience.com>]). This method enabled preservation of stool specimens at ambient temperature for transportation to the laboratory. We offered participants monetary stipends on receipt of adequate stool specimens (\$25 for the first specimen, \$50 for the second, and \$75 for the third).

Microscopic Analysis

Upon receiving the specimens at the UNC laboratory, we homogenized the specimens by using sterile inoculating loops (VWR, <https://us.vwr.com>). We stored formalin-preserved stools at ambient temperature and stored Zn-PVA-preserved stools at 4°C. Trained laboratory technicians used the mini-FLOTAC method (23) to identify and enumerate helminth eggs from formalin-preserved samples (Appendix, <https://wwwnc.cdc.gov/EID/article/29/12/23-0751-App1.pdf>). In brief, we homogenized 4 grams of the stool-formalin mixture with 36 mL of sodium nitrate (VWR) solution (specific gravity 1.25) in a fill-FLOTAC and then dispensed them into 3 mini-FLOTAC disks. After 10 minutes, we turned and read the disks at 100 \times magnification by using a trinocular light microscope (VWR). The theoretical limit of detection of this method was 3.3 eggs/g (24). We photographed suspected eggs by using a mounted camera (Motic, <https://www.motic.com>) and sent images to CDC's DPDx teliagnosis service (<https://www.cdc.gov/dpdx/index.html>) for morphologic confirmation.

Molecular Analysis by TaqMan Array Card and Digital PCR

After homogenization, we extracted nucleic acids from 150 mg of selected Zn-PVA preserved stool by using the QIAamp 96 Virus QIAcube HT Kit (QIAGEN, <https://www.qiagen.com>), which included a pretreatment step using Precellys SK38 bead beating

tubes (Bertin Technologies, <https://www.bertin-technologies.com>) (25–27). We typically extracted samples within 1–4 weeks of receipt (median 15 days, interquartile range [IQR] 8–28 days, range 1–405 days); we extracted 92% of samples within 8 weeks. Among children who submitted >1 stool specimen, we randomly selected a single replicate for extraction. We randomly selected ≈5% of stools for duplicate extraction and another 3% for extraction from multiple replicates. We included ≥1 extraction-negative control (28) during each day of extractions and spiked samples with 10⁷ copies of phage MS2 and 10⁶ gene copies of synthetic DNA (IDT, <https://www.idtdna.com>) as extraction-positive controls. We stored extracts at –80°C until analysis. We assessed extracts from specimens suspected to potentially be from nonhuman sources by using digital PCR (dPCR) (QIAcuity 4; QIAGEN) for human mitochondrial DNA (29).

At the UNC laboratory, we analyzed nucleic acids for 7 helminths by using a custom TaqMan Array Card (TAC) on a Quantstudio 7 Flex (ThermoFisher Scientific, <https://www.thermofisher.com>), following the methods described in Liu et al. (30) The targets included were *Ancylostoma duodenale*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *N. americanus*, *Rodentolepsis (Hymenolepsis) nana*, *S. stercoralis*, and *Trichuris trichiura*. We prepared the TAC by combining 40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents (ThermoFisher Scientific). We evaluated the TAC performance by using an 8-fold dilution series (10⁹–10² gene copies per reaction) of an engineered combined positive control that was developed using the methods from Kodani and Winchell (31). Linearity and efficiency for the six targets were within normative standards (linearity 0.99–1.0, efficiency 95%–100%) (Appendix Tables 1, 2, Figure 1). Each day of TAC analysis, we ran ≥1 positive and negative (either an extraction-negative control or a PCR-negative control). We determined quantification cycle values by manual thresholding and included comparison of each specimen's fluorescent signal against the daily negative and positive controls (Appendix Figure 2). We categorized any target that amplified past a quantification cycle of 35 as negative to reduce the potential for false positives (30).

In addition, we analyzed nucleic acids available from children living in Lowndes and Wilcox counties for *N. americanus* DNA by using dPCR because of its higher sensitivity (Appendix Tables 3, 4, Figure 2). We prepared reactions with QIAcuity Probe Mastermix (QIAGEN) by using 200 nM forward and reverse primers, 800 nM probe, and 4 µL of template. Thermocycling conditions were 95°C for 2 min,

followed by 45 cycles of 95°C for 15 s and 55°C for 60 s. We included ≥1 positive and negative control on each dPCR nanoplate. We set the threshold manually between the bands of the positive and negative controls. We classified specimens with <3 positive partitions as negative (Appendix Table 4).

Molecular Analysis by Multiparallel Quantitative PCR

We aliquoted 2 mL of Zn-PVA stool samples into sterile cryovials, stored them at 4°C, and shipped them to CDC's Division of Parasitic Diseases and Malaria for qPCR analysis. We removed the preservative and extracted DNA from 500 mg stool by using either DNeasy PowerSoil Kit or DNeasy PowerSoil Pro Kit (QIAGEN). Eggs were broken up through bead beating in FastPrep-24 homogenizer (MP Biomedicals, <https://www.mpbio.com>) for 3 min at 6.5 m/s. We performed the DNA extraction procedure in the QIAcube automated nucleic acid purification system (QIAGEN) following the manufacturer's instructions. We quality control tested DNA extracts for presence of potential amplification inhibitors by using a human cytochromeB gene qPCR (32). We tested DNA samples without inhibition by using multiparallel qPCR assays specific for *N. americanus*, *A. duodenale*, *T. trichiura*, *S. stercoralis* (33), and *A. lumbricoides* (34). We performed qPCR reactions in a total volume of 25 µL, consisting of 250 nM of each primer, 125 nM of probe (Platinum Quantitative PCR SuperMix-UDG w/ROX; ThermoFisher Scientific), and 2 µL of DNA template. Each qPCR run was accompanied by positive (genomic DNA from STH worms) and negative (water and DNA extracted from STH-free feces) amplification controls. We performed the qPCR on an AriaMx Real-Time PCR System (Agilent, <https://www.agilent.com>) with the following cycling conditions: 50°C for 2 min, 95°C for 2 min, then 40 cycles of 95°C for 15 s and 59°C for 60 s.

Antibody Detection for *Toxocara* spp. and *S. stercoralis*

We performed detection of antibodies against *Toxocara* spp. and *S. stercoralis* on dried blood spots by using Luminex assay as previously described (35,36). In brief, we placed the dried blood spots in 0.25 mL of elution buffer at 4°C overnight. We allowed antibodies in the eluate to bind to recombinant antigens *T. canis* C-type lectin and 31 kDa third stage *S. stercoralis* larval antigen coupled to beads. We detected bound antibodies by using R-phycoerythrin reporter (ThermoFisher) in a MAGPIX reader with xPONENT software (ThermoFisher). We considered samples positive at >8 median fluorescence intensity for *S. stercoralis* and 23.1 median fluorescence intensity for

Toxocara spp. We determined cutoff points by testing sets of defined positive, negative, and cross-reactive serum samples and analyzing the results by receiver operating characteristics curve. We logged in data from case report forms and laboratory results into REDCap (<https://www.project-redcap.org>) and analyzed the data by using SAS version 9.4 (SAS Institute Inc., <https://www.sas.com>). We estimated combined sensitivity resulting from multiple microscopic and molecular assays by using surrogate canine hookworm (*Ancylostoma caninum*) (Appendix Table 5, Figures 3, 4).

Ethics Considerations

This study was approved by the institutional review boards of the University of Alabama at Birmingham (approval no. 300002219), Georgia Institute of Technology (approval no. H19021), and UNC (approval no. 20-3212). The study was reviewed by CDC and conducted consistent with applicable federal laws and policy.

Results

We enrolled 777 eligible participants from 442 unique households, representing $\approx 10\%$ of the children living in the study area (Tables 1, 2; Figure 1). The higher density of enrollment overlapped with higher density of households. Of enrolled children, 93 (12%) reported living in homes with a straight pipe, discharging untreated sewage in the yard or nearby (Figure 2). A total of 227 participants submitted dried blood spot samples, and 704 participants submitted stool samples; 676 children submitted ≥ 3 separate stool samples. For 169 participants, we collected both blood and stool samples.

Antibody Detection on Dried Blood Spots

Of the 227 dried blood spots analyzed, 8 were inconclusive because of insufficient sample and 11 tested positive for *Toxocara* antibodies, resulting in a positive exposure rate of 5%. None were positive for *S. stercoralis* antibodies.

Control Sample Results for Molecular Detection Methods

For PCR run on the TAC platform at the UNC laboratory, the extraction-positive control consistently amplified (median cycle threshold 18), indicating no inhibition present. We observed no contamination among extraction-negative controls ($n = 19$) or PCR-negative controls ($n = 2$), and our PCR-positive controls ($n = 30$) exhibited the expected amplification for all targets (Appendix Table 2). We observed no contamination among any template controls ($n = 16$) for

dPCR, and positive controls exhibited positive partitions ($n = 14$) (Appendix Table 4). At CDC, 11 DNA extracts (0.6%) showed amplification inhibition and were thus excluded from further testing.

Microscopic Examination and Molecular Results

We observed no STH eggs through microscopic examination on any stool sample received from the 704 eligible children who submitted stool samples to the UNC laboratory. Aliquots from samples with sufficient volume (1,803 stools from 625 children) were also tested at the CDC by multiparallel qPCR assays specific for *N. americanus*, *A. duodenale*, *T. trichiura*, *S. stercoralis*, and *A. lumbricoides*; all results were negative. We randomly selected a subset of samples for additional testing by 2 different molecular methods at the UNC laboratory; we analyzed 1 stool each from 488 children on TAC and 265 on dPCR. We observed *E. vermicularis* eggs in stool from 2 children (0.28% [2/704]) by microscopic examination and detected *E. vermicularis* DNA in 2 samples (0.41% [2/488]) by TAC. We did not detect DNA from *A. duodenale*, *A. lumbricoides*, *H. nana*, *N. americanus*, *S. stercoralis*, or *T. trichiura* by using the TAC platform, and we did not detect DNA from *N. americanus* by using dPCR.

Combined Sensitivity

In recovery experiments using canine hookworm (*Ancylostoma caninum*) (Appendix), for 10% formalin at ambient temperature we observed a 0.005 \log_{10} reduction in egg count per day; for Zn-PVA at ambient temperature we observed a 0.033 \log_{10} in gene copies per day, and at 4°C we observed a 0.015 \log_{10} reduction in gene copies per day (Appendix Figures 3, 4). A 2-week gap typically occurred from sample collection to receipt at the laboratory (median 14 days, IQR 11–21 days); we extracted DNA approximately 2 weeks later (median 15 days, IQR 8–28 days), and we usually performed mini-FLOTAC within 2 weeks of receipt (median 13 days, IQR 4–28 days). The 95% limits of detection were 4.0 gene copies/ μL template for the *N. americanus* qPCR assay and 0.43 gene copies/ μL template for the dPCR assay (Appendix Figure 1). In addition, we estimated that a single undeveloped *A. caninum* ova on average contained 2,220 gene copies of our target sequence (Appendix Figure 4).

Considering this time-dependent reduction in targets (i.e., eggs and DNA), we calculated the estimated sensitivity by assay and the combined sensitivity for a single child shedding 1–100 eggs/g of stool (Table 3). We estimated 100% combined sensitivity to detect hookworm eggs at a concentration of 7 eggs/g (accounting for recovery), which is at the low end of a

Table 1. Characteristics of 777 participants based on self-administered surveys conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*

Characteristic	No. (%)	No. missing
Age, y, mean (SD), median (range)†	10.6 (4.4), 11 (2–18)	7
Years living in current house, mean (SD), median (range)	8.1 (4.8), 8.0 (0–18.0)	18
Sex		
F	393 (50.8)	4
M	380 (49.2)	
Race		
Black or African American	734 (95.2)	6
White	21 (2.7)	
Unknown	2 (0.3)	
Prefer not to answer	14 (1.8)	
Ethnicity		
Hispanic or Latino	11 (1.6)	89
Not Hispanic or Latino	635 (92.3)	
Unknown	6 (0.9)	
Prefer not to answer	36 (5.2)	
County of residence		
Wilcox	352 (45.3)	
Lowndes	132 (17.0)	
Perry	293 (37.7)	
Animals		
Dogs	331 (43.3)	13
Cats	121 (15.8)	
Pigs	7 (0.9)	
None	380 (49.7)	
Other (horse, chicken)	10 (1.3)	
Contact with soil		
Never	233 (30.3)	8
Less than once a month	323 (42.0)	
At least monthly	191 (24.8)	
Not sure	22 (2.9)	
Eat produce from home garden		
Yes	357 (46.7)	13
No	407 (53.3)	
Traveled outside the United States in past 5 y		
Yes	14 (1.8)	13
No	750 (98.2)	
Sewer connection	227 (29.6)	11
Septic tank	312 (40.7)	
Cess pit	2 (0.3)	
Straight-pipe	94 (12.3)	
Don't know	125 (16.3)	
Other	6 (0.8)	
Sewage contamination of property in the past year		
Yes	62 (8.4)	35
No	680 (91.6)	
If yes, where was the contamination?		
Inside the house	13 (24.5)	9
In the yard	40 (75.5)	
Payment of water bill		
Yes	643 (83.3)	5
No	123 (15.8)	
Don't know	6 (0.8)	
Amount of screen time daily		
<2 h	128 (16.8)	13
2–4 h	336 (44.0)	
>4 h	300 (39.3)	
Believe screen time prevents child from playing outdoors		
Yes	179 (23.6)	20
No	578 (76.4)	
No. stools received		
0	73 (9.4)	
1	8 (1.0)	
2	20 (2.6)	
3	676 (87.0)	

*Values are no. (%) except as indicated.

†Age was calculated on the basis of time between (self-reported) date of birth and date of form completion.

Table 2. Percentage of children enrolled, by age group and race per county population, in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*

Characteristic	Lowndes County	Perry County	Wilcox County
Age group, y			
<5	1.5	7.6	2.2
5–9	5.1	13.8	9.8
10–14	9.1	14.8	24.8
15–19	5.5	8.2	14.1
Race			
Black or African American	6.4	12.6	16.5
White	1.6	0.7	1.7

*Based on 2020 US Census data.

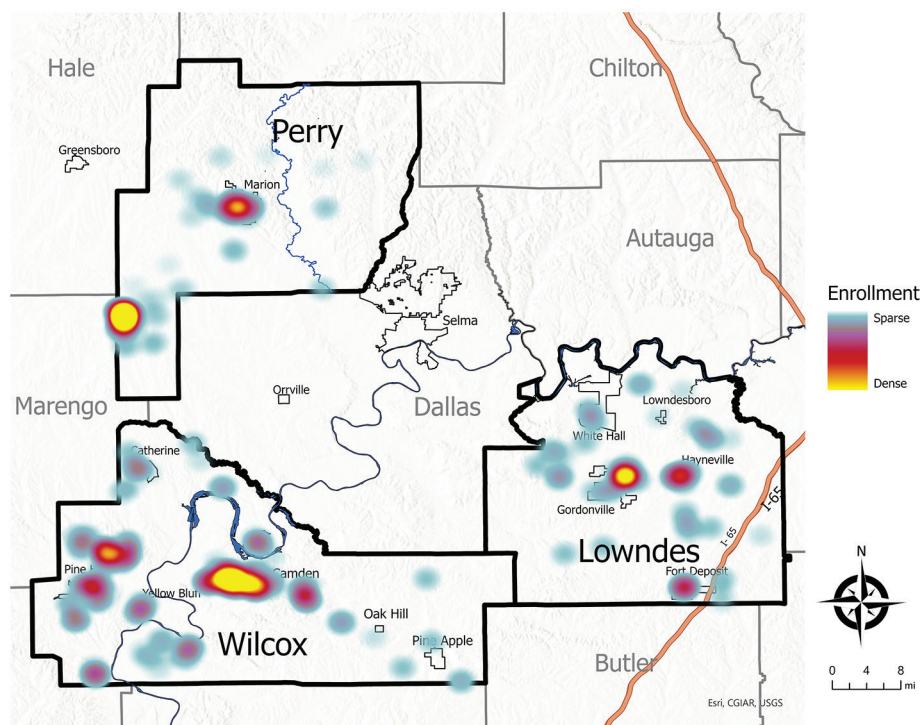
light infection as defined by the World Health Organization (i.e., 1–1,999 eggs/g) (37). We also estimated assay and combined sensitivity without considering recovery to demonstrate the theoretically ideal performance of our methods. Not accounting for recovery, we estimated 100% combined sensitivity at a concentration of 3 eggs/g (Table 3).

Discussion

Our survey findings confirmed that a substantial number of homes in our study region lack adequate sanitation, resulting in potential exposure of children to untreated sewage. However, we did not identify any cases of STH, a finding in contrast to McKenna et al. (17), who reported 19 cases of *N. americanus* infection and 4 cases of *S. stercoralis* infection among 55 persons in Lowndes County. They detected cases through qPCR at very low concentration by using a standard curve from a previous study, translating to

an estimated mean burden of 1–2 eggs/g. Subsequent microscopic examination of specimens from 9 of the 19 positive persons by the Alabama Department of Public Health and the CDC did not detect any hookworm eggs. *Toxocara* seroprevalence was higher in our Alabama cohort than in the national study in comparable age ranges (3.0% in ages 6–11 years, 3.9% in ages 12–19 years) (38), indicating higher levels of exposure in the American Southeast, as is also demonstrated in a recent Mississippi surveillance study (22). Detection of *E. vermicularis* pinworms in stool samples from our cohort was rare.

There are several factors to consider why our results differ to the McKenna et al. (17) study. In the McKenna et al. study, participants were mostly adults and were tested in 2013. In endemic populations, the prevalence of hookworm and *S. stercoralis* threadworm increases with age (39,40) because adult worms can live in the gut for several years (41); thus,

**Figure 1.** Heat map demonstrating home location distribution of children enrolled in a study of soil-transmitted helminthiasis conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022.

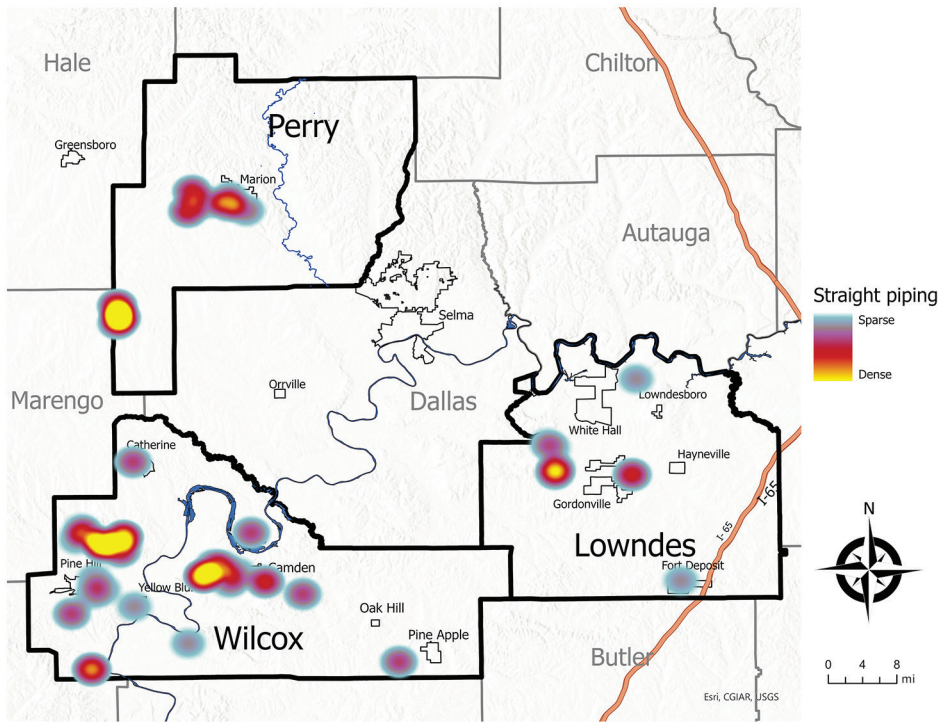


Figure 2. Heat map demonstrating distribution of children enrolled living in homes with self-reported straight pipe sewage discharge in a study of soil-transmitted helminthiases conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022.

although residual infections were reported by McKenna et al., transmission may have since ceased. We only enrolled children because they are most at risk for adverse outcomes associated with STH infection, including anemia (42), cognitive deficits, potential growth faltering (43), and other outcomes (44). In addition, our studies used different methods for sample preservation before analysis; the McKenna et al. study processing stool stored initially on dry ice for up to 5 days, followed by storage until analysis at -20°C (time from collection until analysis not reported).

Conclusive evidence on whether endemic human hookworm exists in rural Alabama would be the identification of a case according to standard diagnos-

tic criteria (observation of ≥ 1 definitive hookworm eggs by microscopic examination of a stool specimen), without the possibility of having acquired the infection outside Alabama. To our knowledge, such evidence has not been demonstrated in the recent past. A review of Medicaid claims data from 2010–2018 indicated that STH infections continue to be clinically diagnosed in children in Alabama, but rarely (45). Without confirmatory stool diagnostic data, drawing conclusions regarding ongoing transmission is difficult because such diagnoses are frequently made empirically on the basis of parental reports of seeing worms in the stool. In 1991, microscopic examination of stool samples collected from children in Wilcox

Table 3. Estimated sensitivity to detect STH infection by assay method in a single infected child for different assumed intensity infections (for fecal testing methods used in a STH prevalence study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*)

Egg/g feces from 1 child	Mini-FLOTAC, triplicate, %	qPCR, single, %	dPCR, single, %	Combined, %
Sensitivity accounting for recovery				
1	2	2	18	20
3	5	6	53	55
5	9	10	89	90
7	12	14	100	100
10	17	20	100	100
100	93	100	100	100
Sensitivity not accounting for recovery				
1	2	10	90	91
3	7	30	100	100
5	12	51	100	100
7	17	71	100	100
10	23	100	100	100
100	99	100	100	100

*dPCR, digital PCR; qPCR, quantitative PCR.

County identified 3 cases of *A. lumbricoides* infection out of 81 samples collected (16). The last published population-based survey using microscopic examination to identify STH eggs in stool samples in the United States found a single positive case of hookworm in a sample of 561 children 3–7 years of age in Kentucky in 1982 (46).

Sustained hookworm transmission requires 3 factors: infected persons shedding eggs; environmental conditions for eggs to mature into larvae, typically in sandy soil where temperature and moisture conditions are favorable (47); and exposure to susceptible new hosts through contact of the larvae with skin (47). In settings with endemic hookworm transmission, studies indicate that some persons within a population shed large numbers of eggs, sufficient to maintain transmission to others, whereas other persons may have moderate- or low-intensity infections (47,48). If hookworm were endemic to this region, we would expect to have identified some cases with microscopically detectable hookworm eggs. The negative results from microscopic examination were concordant with more sensitive qPCR and dPCR assays we performed on a subset of samples. In addition, we analyzed triplicate samples from 129 persons from Lowndes County, in contrast to McKenna et al. (17), who tested single samples from only 55 persons (48,49). Whereas our survey possibly could have missed isolated infections in this population, we do not consider that result likely given what is known about endemic hookworm transmission. We estimate high combined sensitivity for light infections (<100 eggs/g) in the subset of participants that was tested with all methods.

In conclusion, our study did not confirm endemic STH infection in the Alabama Black Belt. However, our survey data suggests a considerable number of children in this region may be exposed to raw sewage, which is itself a major public health concern.

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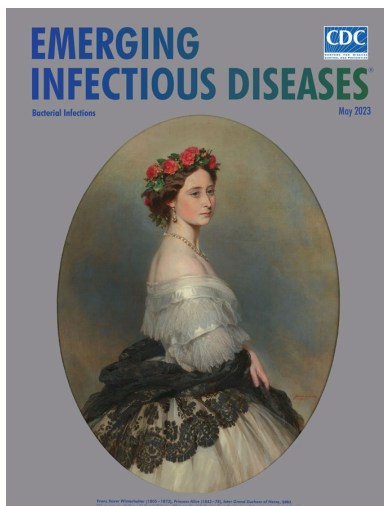
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- Phylogenetic Analysis of Transmission Dynamics of Dengue in Large and Small Population Centers, Northern Ecuador
- Emergence of Erythromycin-Resistant Invasive Group A *Streptococcus*, West Virginia, USA, 2020–2021
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- Misdiagnosis of *Clostridioides difficile* Infections by Standard-of-Care Specimen Collection and Testing among Hospitalized Adults, Louisville, Kentucky, USA, 2019–2020
- SARS-CoV-2 Seroprevalence Compared with Confirmed COVID-19 Cases among Children, Colorado, USA, May–July 2021
- Disparities in Implementing COVID-19 Prevention Strategies in Public Schools, United States, 2021–22 School Year
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- Influence of Sex and Sex-Based Disparities on Prevalent Tuberculosis, Vietnam, 2017–2018



- Cutaneous Leishmaniasis Caused by *Leishmania infantum*, Israel, 2018–2021
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- Severe *Streptococcus equi* Subspecies *zooepidemicus* Outbreak from Unpasteurized Dairy Product Consumption, Italy
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- Case–Control Study of Long COVID, Sapporo, Japan
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- Spatiotemporal Evolution of SARS-CoV-2 Alpha and Delta Variants during Large Nationwide Outbreak of COVID-19, Vietnam, 2021
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**EMERGING
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Cross-Sectional Study of Soil-Transmitted Helminthiases in Black Belt Region of Alabama, USA

Appendix

Microscopy Training

Author DC first received training on helminth identification and enumeration from staff at the Mozambican National Institute of Health's Parasitology Lab in Maputo, Mozambique. Second, author DC received training in mini-FLOTAC and helminth identification and enumeration from the Kaplan Lab at the University of Georgia, which also serves as the U.S. Distributor for mini-FLOTAC.

All laboratory technicians were trained by DC in helminth identification and enumeration, except author TB who previously worked as a technician in a veterinary parasitology laboratory. Laboratory technicians were trained over a period of 2 to 4 weeks. Technicians were required to read the following references: 1) World Health Organization's "Bench Aid for the Diagnosis of Intestinal Parasites" (First and Second Editions); 2) CDC DPDx's "Diagnostic Procedures for Intestinal Parasites" (<https://www.cdc.gov/dpdx/diagnosticprocedures/stool/morphcomp.html>); 3) CDC DPDx's "Artifact Identification Sheet" (<https://www.cdc.gov/dpdx/artifacts/index.html>); 4) Donald L. Price's "Procedure Manual for the Diagnosis of Intestinal Parasites"; and 5) Ash and Orihel's "Human Parasitic Diseases: A Diagnostic Atlas." Then, technicians were trained for 1 day on using a microscope and practiced identifying ova that were fixed and mounted onto prepared slides (VWR, Radnor, PA). The study team acquired feces from dogs, cats, chickens, pigs, horses, and cows that contained a wide range of helminth ova (e.g., hookworm, *Ascaris*, *Trichuris*, *Toxocara*, *strongyloides*, and pinworm) and artifacts (e.g., pollen, undigested food,

and mite eggs). Technicians received a day of instruction on sodium nitrate solution preparation and the mini-FLOTAC method from either DC or TB, and continued a self-guided period of training with different stool samples for 2–3 weeks. Frozen human stool samples collected as part of the MapSan Trial (1) that contained *Ascaris* and *Trichuris* ova were used in training. As the final step in training lab technicians had to demonstrate the ability to enumerate ova within 25% of the count observed by DC. Finally, during the analysis phase of the study, technicians consulted DC and TB for help identifying ova if they were uncertain.

Combined Sensitivity

Mini-FLOTAC Sensitivity

We took a highly conservative approach to estimate the sensitivity of mini-FLOTAC. First, we consulted Cools *et al.* 2019 (2), which in Figure 2 reports a sensitivity of 21% for 0–49 eggs per gram and 82% for 50–149 eggs per gram. As these were reported as ranges, we assumed that these values best represented the sensitivity of the median value in the range, which were 25 and 100 respectively. We fit a linear regression line (slope = 0.0081) between these two values to interpolate the sensitivity for egg per gram values between 25 and 100. Assuming the sensitivity of stool with no ova is 0, we used the same methodology to interpolate individual sensitivities (slope = 0.0084) from 0 to 25 ova per gram. For 1 ovum per gram the calculation is as follows:

$$\text{Eq 1. } \text{Sensitivity}_n = \text{Sensitivity}_{25} - \left(\frac{21\%}{25}\right) * (25 - n) = 21 - \left(\frac{21\%}{25}\right) * (25 - 1) = 21 - 20.16 = 0.84\%$$

We also accounted for degradation of the ova in 10% formalin. In Appendix Figure S3 we found that egg counts reduced by 0.0049 log₁₀ per day on average. The median number of days between sample collection and analysis was 28. This suggests that if a stool sample contained ova, the concentration would have decreased by 0.14 log₁₀ from collection to analysis. We subtracted this value from the initial eggs per gram, and then used this value to calculate the sensitivity after considering egg degradation. For example, if 1 ova per gram was initially present in the stool, the concentration following preservation would be:

$$\text{Eq 2. } \text{Concentration}_n = \text{Concentration}_0 - 0.14 \log_{10} = 0 \log_{10} - 0.14 \log_{10} = -0.86 \log_{10} = 10^{-0.14} = 0.73 \text{ ova per gram}$$

Then we re-calculated the $Sensitivity_n$ for the concentration adjusted to reflect egg degradation in 10% formalin. Replacing n in equation 1 with 0.73, instead of 1, we calculated the sensitivity of a single mini-FLOTAC test to be 0.61% for stool that contained 1 ovum initially upon defecation. However, we did the analysis in triplicate. We calculated the sensitivity of this triplicate analysis with equation 3, which reports the sensitivity of 1 egg per gram.

$$\text{Eq 3. } \text{Sensitivity}_{\text{triplicate},n} = 1 - (1 - \text{Sensitivity}_{\text{single}})^3 = \text{Sensitivity}_{\text{triplicate},1} = 1 - (1 - 0.61)^3 = 1.8\%$$

We repeated this methodology to calculate the sensitivity of each integer value from one to a hundred.

qPCR and dPCR Sensitivity

We first quantified the number of gene copies in a hookworm ovum to determine the sensitivity of our molecular methods. We collected three fecal samples from canines infected with *Ancylostoma caninum* at a local animal hospital. Then we enumerated the number of eggs per gram by performing mini-FLOTAC in triplicate. Next, we extracted total nucleic acids from 100 mg of each stool sample in triplicate. Finally, we quantified gene copies of *Ancylostoma caninum* using digital PCR (QIAcuity 4, Qiagen, Hilden, and Germany). Dividing the number of gene copies by the number of ova per stool indicated a mean of 2,220 gene copies per ovum (IQR = 437, 3600).

Next, we considered the dilution during sample processing. The dilutions used would have required 5,500 gene copies and 825 gene copies, for qPCR and for dPCR respectively, of the target sequence present per gram of feces for one gene copy to be theoretically present in the respective PCR reaction. However, one gene copy is unlikely to consistently amplify in a PCR reaction. We accounted for this by analyzing replicates of low concentrations (e.g., 10^{-1} , 10^0 , 10^1 , 10^2 copies per μL) of an engineered plasmid (3) to determine the 95% limit of detection (LOD) using the methods described in Stokdyk et al. 2016 (Appendix Figure 1) (4). We determined the concentration of the plasmid based on the mass of an individual plasmid and the total quantity of DNA provided by the manufacturer (GeneArt ThermoFisher Scientific,

Waltham, Massachusetts). With these methods we determined the 95% LOD for qPCR was 4.0 gene copies per uL template and for dPCR was 0.40 gene copies per uL template. In our dPCR reaction, we used four uL of template, which suggests the 95% LOD was 1.6 gene copies per reaction. However, we required three positive partitions for a sample to be considered positive following manual thresholding based on the performance of our negative controls (Appendix Table 3, Appendix Table 4). We accounted for this by substituting the calculated 95% LOD for dPCR – which was 1.6 gene copies per reaction – with 3.0 gene copies per reaction in our sensitivity calculation and allow for a more conservative estimate. Combining the estimated LOD with our dilutions, we determined there was a 95% chance of detecting hookworm DNA at concentrations of 21,896 gene copies per gram stool for qPCR and 2,475 for dPCR.

Similar to our methods for mini-FLOTAC, we also considered the potential decay of DNA in the Zn-PVA preservation buffer between sample collection and analysis. Using the canine feces described previously to quantify the number of gene copies of *Ancylostoma caninum* per ovum, we aliquoted these feces into different preservation buffers under different storage conditions and extracted nucleic acids over time (Appendix Figure 4). The results indicated a 0.033 log₁₀ reduction in the concentration of hookworm DNA per day in ZnPVA at ambient conditions and a 0.015 log₁₀ reduction at 4°C. We tracked the time between sample collection, receipt, and analysis. There was a median of 14 days at ambient conditions, which occurred before receipt at the lab, and a median of 15 days for storage at 4°C before analysis. This combined decay suggests the initial concentration of hookworm DNA would have decreased by 0.70 log₁₀ from sample collection to analysis. Combining our 95% LODs with the estimated decay enables an estimate of what initial concentration would have been necessary to detect hookworm DNA using our methods. This is demonstrated in Equation 4.

$$\text{Eq 4. } \log_{10} \text{ Gene Copies}_{decay} = \log_{10} \text{ Gene Copies}_{LOD} + 0.70$$

The equation indicates that for qPCR 110,322 gene copies and for dPCR 12,470 gene copies would have needed to be present per gram of feces upon sample collection to have a 95% chance of positive detection. Given that there are 2,200 gene copies per hookworm ova, the methods provided a 95% chance of detecting 47.2 ova per gram via qPCR and 5.3 ova per gram via dPCR. The individual sensitivities are then calculated by Equation 5.

$$\text{Eq 5a. } \text{Sensitivity}_{n,qPCR} = n * \frac{2,200}{110,322}$$

$$\text{Eq 5b. } Sensitivity_{n,dPCR} = n * \frac{2,200}{12,470}$$

The combined sensitivity was calculated using equation 6.

$$\text{Eq 6. } Sensitivity_{overall} = 1 - (1 - Sensitivity_{FLOTAC})(1 - Sensitivity_{qPCR})(1 - Sensitivity_{dPCR})$$

Geospatial Map methodology

Geospatial maps were created using geographic information system GIS software (Esri ArcPro 2.8). Participant residential address locations with attributes related to well water and sanitation type were geocoded using ArcPro and Esri StreetMap Premium. Maps were cartographically designed to maintain participant privacy using heat maps to display general distribution rather than exact locations.

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Appendix Table 1. TAC performance of assay used in study conducted in Alabama, USA, December 2019–August 2022

Target	Target Gene	R ²	Efficiency	Reference
<i>Ancylostoma duodenale</i>	ITS-2	1.000	98%	(5)
<i>Ascaris lumbricoides</i>	ITS-1	1.000	95%	(5)
<i>Enterobius vermicularis</i>	5S	0.999	95%	(6)
<i>Hymenolepis nana</i>	ITS-1	1.000	98%	(7)
<i>Necator americanus</i>	ITS-2	1.000	98%	(5)
<i>Strongyloides stercoralis</i>	Dispersed repetitive sequence	0.999	100%	(5)
<i>Trichuris trichiura</i>	18S rRNA	1.000	99%	(5)

Appendix Table 2. MIQE Checklist for TAC analysis of fecal samples from children enrolled in Lowndes, Perry, and Wilcox Counties in a study conducted in Alabama, USA, December 2019–August 2022

Item to check	Importance	Checklist
Experimental design		
Definition of experimental and control groups	E	There were no experimental or control groups.
Number within each group	E	We ran stool from 488 children on the custom TAC via RT-qPCR.
Sample		
Description	E	Children's stool samples preserved in Zn-PVA
Processing procedure	E	Described in the methods section
Sample storage conditions and duration (especially for FFPE samples)	E	Described in the results section
Nucleic acid extraction		
Procedure and/or instrumentation	E	QIAamp 96 Virus QIAcube HT Kit on a QIAcube HT
Name of kit and details of any modifications	E	We mixed 150 mg of the stool ZnPVA mixture with 1 mL of Qiagen Buffer ASL in Precellys® SK38 bead beating tubes, vortexed to bead beat for five minutes, incubated at room temperature for 15 min, centrifuged at 14000 rpm for 2 min, and then transferred 200 uL of supernatant to the QIAcube to proceed with extraction using the manufacturer's default procedure for the QIAamp 96 Virus QIAcube HT Kit.
Details of DNase or RNase treatment	E	None
Contamination assessment (DNA or RNA)	E	We included one negative extraction control on each day of extractions.

Item to check	Importance	Checklist
Nucleic acid quantification	E	We measured nucleic acids using qubit on a subset of samples
Instrument and method	E	Qubit 4 Fluoremeter, 1X dsDNA High Sensitivity
RNA integrity method/instrument	E	Not performed
Inhibition testing (Cq dilutions, spike or other)	E	We spiked in a DNA and RNA control into each extraction (see methods section).
Reverse transcription		
Complete reaction conditions	E	One-step reverse transcription
Amount of RNA and reaction volume	E	We combined 40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents. The reaction volume was 1.5 µL.
Priming oligonucleotide (if using GSP) and concentration	E	Applied Biosystems, AgPath-ID One-Step RT-PCR Reagents Catalog number: 4387391
Reverse transcription and concentration	E	ArrayScript Reverse transcription
Temperature and time	E	45°C for 20 min
qPCR target information		
If multiplex, efficiency and LOD of each assay.	E	Appendix Table 1
<i>In silico</i> specificity screen (BLAST, etc)	E	We BLASTed all assays to confirm specificity before ordering the custom TAC.
qPCR oligonucleotides		
Primer sequences	E	Citations for primer and probe sequences are listed in Appendix Table 1.
Location and identity of any modifications	E	None
qPCR protocol		
Complete reaction conditions	E	45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min
Reaction volume and amount of cDNA/DNA	E	100µL reactions were prepared, containing 60uL of mastermix and 40µL of template. This corresponds S12 to 0.6µL of template and 0.9µL of mastermix per reaction well.
Primer, (probe), Mg++ and dNTP concentrations	E	All assays contained the same concentrations of primers (900 nmol/L) and probe (250 nmol/L). The Mg2+ and dNTP concentrations are not listed in the in the User Guide.
Polymerase identity and concentration	E	AmpliTaq Gold polymerase
Buffer/kit identity and manufacturer	E	AgPath-ID One-Step RT-PCR Reagents
Additives (SYBR Green I, DMSO, etc.)	E	None
Complete thermocycling parameters	E	45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min
Manufacturer of qPCR instrument	E	ThermoFisher Scientific
qPCR validation		
Specificity (gel, sequence, melt, or digest)	E	See references listed in Appendix Table 1.
PCR efficiency calculated from slope	E	See Appendix Table 1
r2 of standard curve	E	See Appendix Table 1
Evidence for limit of detection	E	See Appendix Figure 1
If multiplex, efficiency and LOD of each assay.	E	All assays were singleplex
Data analysis		
qPCR analysis program (source, version)	E	QuantStudio Real-Time PCR Software V1.2 CDC
Cq method determination	E	Manual thresholding
Results of NTCs	E	For PCR run on the TAC platform, we did not observe contamination among extraction negative controls (n = 19) or PCR negative controls (n = 2), and our PCR positive controls (n = 30) exhibited the expected amplification for all targets. For dPCR we did not observe contamination among NTCs (n = 16) and positive controls exhibited positive partitions (n = 14).
Description of normalization method	E	Mass of stool extracted from (150 mg)
Number and stage (RT or qPCR) of technical replicates	E	Explained in the corresponding publication Capone et al.

Appendix Table 3. dMIQE Checklist for dPCR assay to detect *N. americanus* in fecal samples from children enrolled in Lowndes and Wilcox County in a study conducted in Alabama, USA, December 2019–August 2022

Item to check	Provided, Y/N	Comment
1. Specimen		
Detailed description of specimen type and numbers	Y	We ran Zn-PVA preserved stool from 265 children
Sampling procedure (including time to storage)	Y	Described in methods section
Sample aliquotation, storage conditions and duration	Y	Described in results section
2. Nucleic acid extraction		
Description of extraction method including amount of sample processed	Y	We mixed 150 mg of the stool ZnPVA mixture with 1 mL of Qiagen Buffer ASL in Precellys® SK38 bead beating tubes, vortexed to bead beat for five minutes, incubated at room temperature for 15 min, centrifuged at 14000 rpm for 2 min, and then transferred 200 uL of supernatant to the QIAcube to proceed with extraction using the manufacturer's default procedure for the QIAamp 96 Virus QIAcube HT Kit.
Number of extraction replicates	N	None in dPCR
Extraction blanks included	N	N/A
3. Nucleic acid assessment and storage		
Method to evaluate quality of nucleic acids	N	Not performed
Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass)	Y	We measured nucleic acids using qubit on a subset of samples
Storage conditions: temperature, concentration, duration, buffer, aliquots	Y	Described in the results section
Clear description of dilution steps used to prepare working DNA solution	Y	None
4. Nucleic acid modification		
Template modification (digestion, sonication, pre-amplification, bisulphite etc.)	N/A	None performed
Details of repurification following modification if performed	N/A	None performed
5. Reverse transcription	N/A	None performed
6. dPCR oligonucleotides design and target information		
Sequence accession number or official gene symbol	Y	MH665842.1
Method (software) used for design and <i>in silico</i> verification	Y	NCBI BLAST
Location of amplicon	Y	453 to 474
Amplicon length	Y	102
Primer and probe sequences (or amplicon context sequence)**	Y	5' ->3' Fwd: CTGTTTGTGGAACGGTACTTGC Rev: ATAACAGCGTGACATGTTGC Probe: 56FAM/CTGTACTACGATTGTATAC/3MGB-NFQ
Manufacturer of oligonucleotides	Y	(IDT, Coralville, IA)
7. dPCR protocol		
Manufacturer of dPCR instrument and instrument model	Y	QIAGEN QIAcuity Four machine (Qiagen, Hilden, Germany)
Buffer/kit manufacturer	Y	QIAcuity Probe PCR Kit (5 ml) (Qiagen, Hilden, Germany) Cat. No. / ID: 250102
Primer and probe concentration	Y	Probe: 400nM, Primers: 800nM
Pre-reaction volume and composition	Y	2µL template
Template treatment (initial heating or chemical denaturation)	N/A	None
Polymerase identity and concentration, Mg++ and dNTP concentrations***	N/A	Proprietary (QIAcuity Probe PCR Kit) (Qiagen, Hilden, Germany)
Complete thermocycling parameters	Y	1 × 95°C for 2 min 40 cycles x 95°C for 15 s, 50°C for 60 s
8. Assay validation		
Details of optimization performed	Y	This assay was optimized for our QIAcuity Four dPCR platform by systematically titrating probe and primer concentrations at varying annealing temperatures, aimed at maximizing separation between positive and negative bands and minimizing background noise (i.e., rain). First, primer concentrations of 400, 800, and 1600nM were tested at annealing temperatures of 50°C, 55°C, and 60°C. Next,

Item to check	Provided, Y/N	Comment
Analytical sensitivity/LoD and how this was evaluated	Y	probe concentrations of 200, 400, and 800nM were assessed. The primer, probe, and temperature combination with the greatest reaction efficiency was then selected. See Appendix Figure 1
9. Data analysis		
Comprehensive details negative and positive of controls (whether applied for QC or for estimation of error)		See "Controls" in "Results"
Partition classification method (thresholding)	Y	Threshold manually set to 100 RFU
Examples of positive and negative experimental results (including fluorescence plots in supplemental material)	N	All samples and NTCs ran on the QIAcuity Nanoplate 26k 24-well returned negative experimental results. We observed positive band hits for the positive control.
Description of technical replication	Y	6 samples were randomly selected to be run in duplicate, with all duplicates returning the same negative experimental results as the original samples. Duplicates were run using the same methodology from the same sample aliquots as the originals indicating reproducibility.
Plate type	Y	QIAcuity Nanoplate 26k 24-well (Qiagen, Hilden, Germany)
dPCR analysis program (source, version)	Y	QIAcuity software suite version 1.2 (Qiagen, Hilden, Germany).
Description of normalization method	Y	Mass of stool extracted from (150 mg)

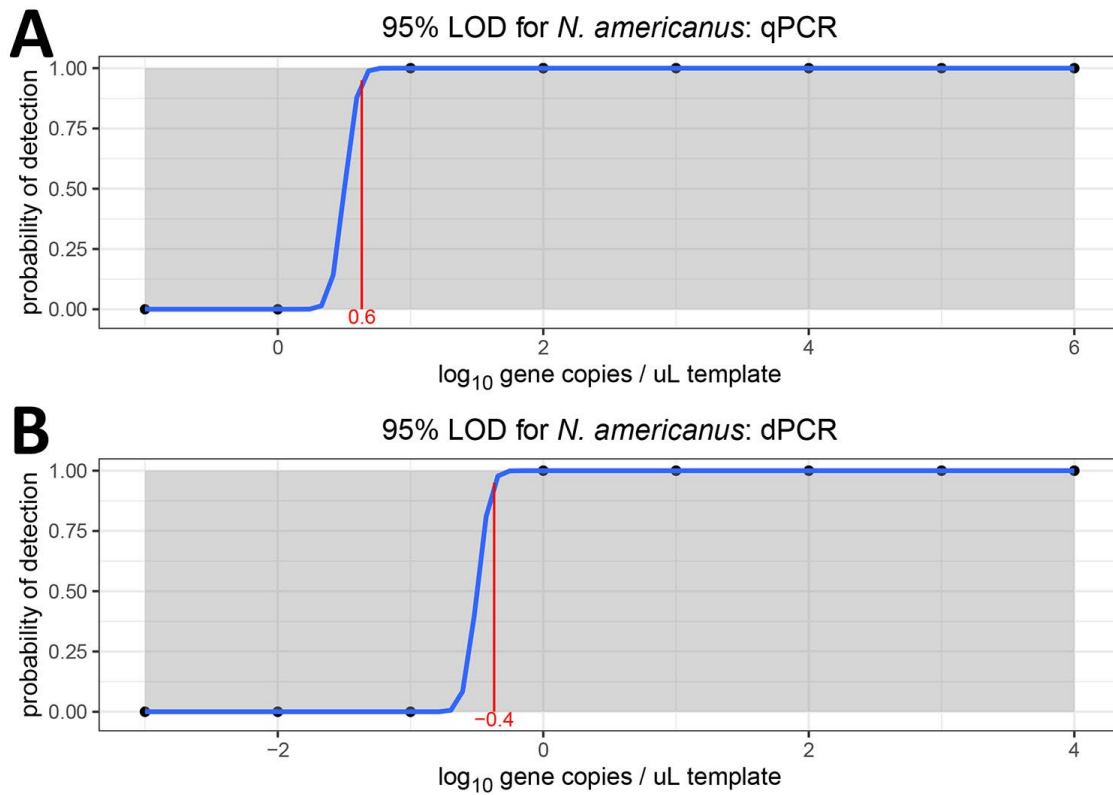
Appendix Table 4. *N. Americanus* dPCR Data Summary of fecal samples from children enrolled in Lowndes and Wilcox Counties in a study conducted in Alabama, USA, December 2019–August 2022

Positive Controls	Value
Total Number Assayed	14
Average valid partitions per sample	24,433
Average positive partitions per sample	7,725
Samples with ≥ 3 partitions positive	14/14
Negative Controls	
Total Number Assayed	16
Average valid partitions per sample	25,412
Average positive partitions per sample*	0.25
Samples with ≥ 3 partitions positive	0/16
Stool Samples	
Total Number Assayed	265
Average valid partitions per sample	25,415
Average positive partitions per sample *	0.06
Samples with ≥ 3 partitions positive	0/265
Number of duplicate samples analyzed	30

*Among our 16, no template controls two had one positive partition and one had two positive partitions. Based on this data, and best practice with digital PCR, we only considered samples positive if three or more partitions were above the line of manual thresholding (Appendix Figure 2).

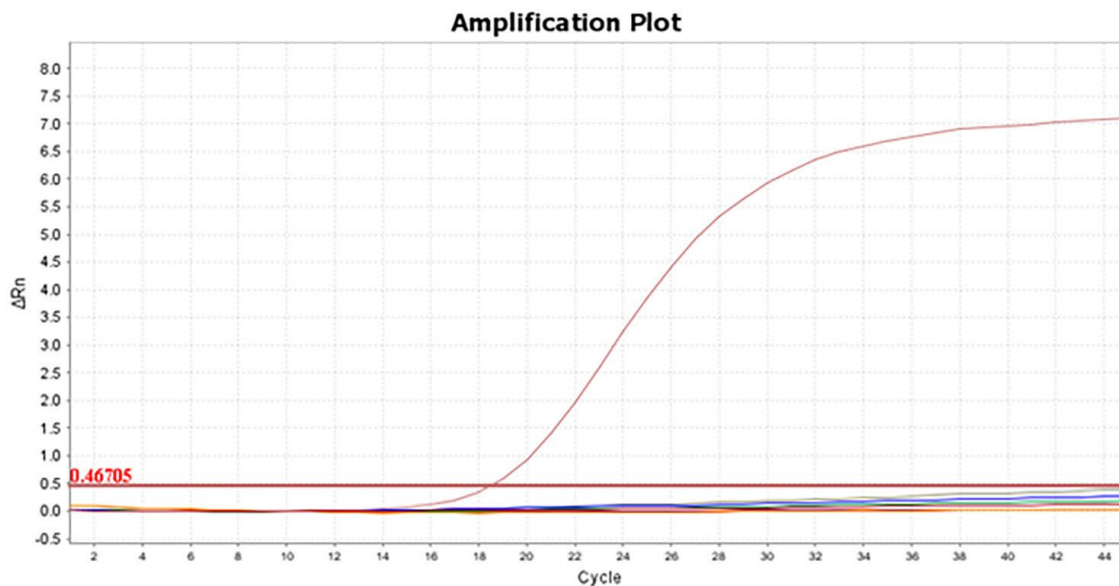
Appendix Table 5. Decay constants for *Ancylostoma caninum* DNA in different preservation buffers

Target	Preservative	log ₁₀ decay of DNA per day
<i>Ancylostoma caninum</i>	Zn PVA (4°C)	-0.0147
	Zn PVA (20°C)	-0.0331
	UNEX	-0.0079
	TotalFix	-0.0267
	NAP	-0.0010
	70% Ethanol	-0.0228

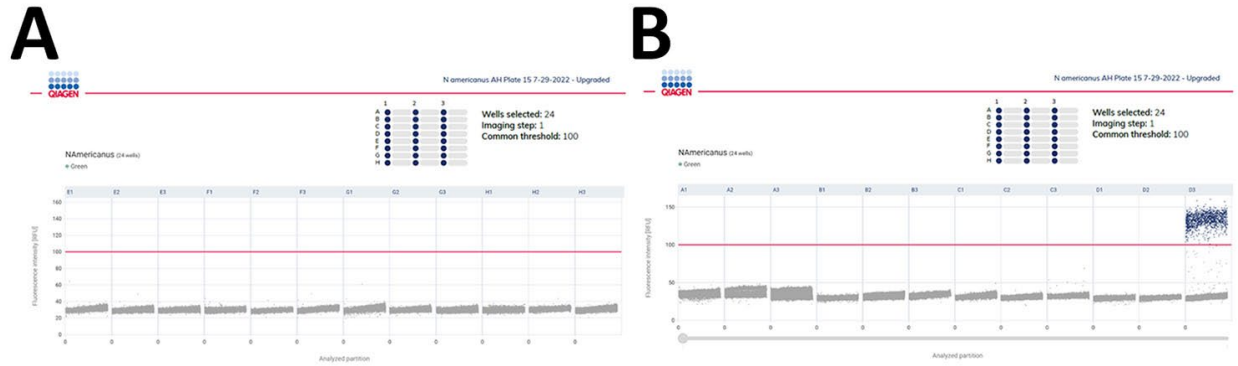


Appendix

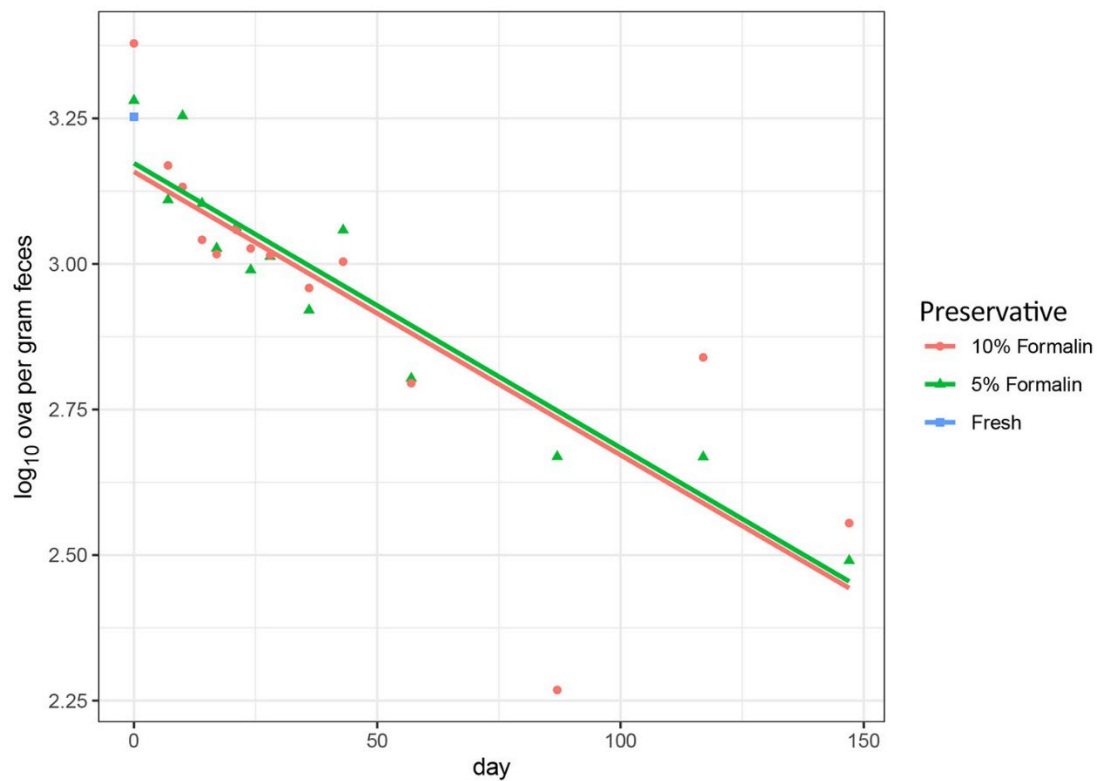
Figure 1. 95% Limit of detection for *N. americanus*: qPCR for assay used in study conducted in Alabama, USA, December 2019–August 2022.



Appendix Figure 2. Amplification and Multicomponent Plots used to determine quantification cycle for TAC analysis used in study conducted in Alabama, USA, December 2019–August 2022. The positive control amplified but the negative control and samples do not.

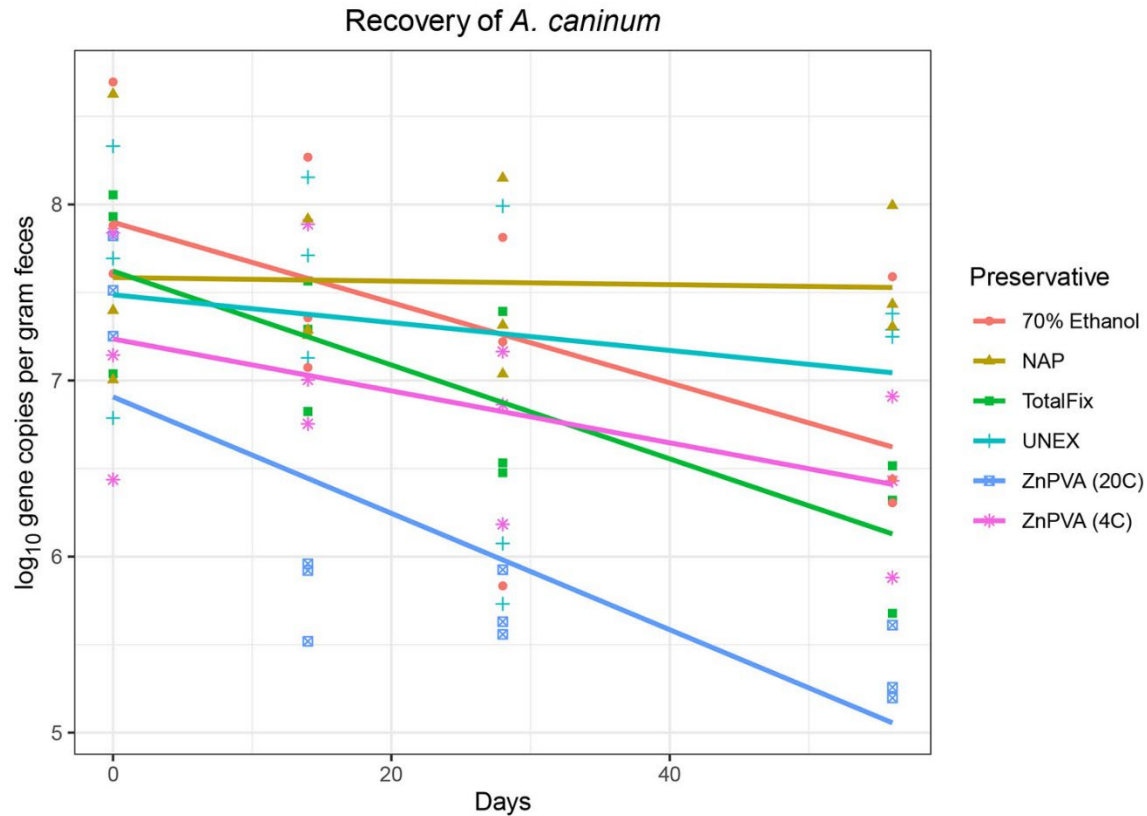


Appendix Figure 3. Screenshots from a nanoplate run for *Necator Americanus* by dPCR assay used in study conducted in Alabama, USA, December 2019–August 2022. All samples were negative except the positive control.



Appendix Figure 4. Recovery Experiment of *Ancylostoma caninum* from canine feces in 10% and 5% formalin over time. We received canine feces containing *Ancylostoma caninum* from the Kaplan Lab in the College of Veterinary Medicine at the University of Georgia. Upon receipt, we aliquoted and homogenized a portion of the stool 1:1 into 10% Formalin and into 5% Formalin. Then we enumerated the fresh (2 g) and preserved stool (4 g of the Formalin-Stool mixture) using mini-FLOTAC in triplicate. The

preserved stool was stored at room temperature and hookworm ova were enumerated weekly, and then monthly, over a period of 5 months. We observed a loss of 0.0049 log₁₀ ova per day in both 5% and 10% Formalin.



Appendix Figure 5. Nucleic Acid Recovery Experiment using *Ancylostoma caninum* comparing different fecal sample preservatives. We collected feces from three dogs at an animal hospital in rural North Carolina, enumerated hookworm ova using mini-FLOTAC and confirmed the species was *Ancylostoma caninum* using dPCR. We combined aliquots of the feces 1:1 with five different preservatives: ZnPVA at 4°C, ZnPVA at ambient (i.e., 20°C), 70% ethanol, TotalFix, homemade Nucleic Acid Preservation Buffer (NAP, Camacho-Sanchez *et al.* 2013) (8), and homemade Universal Extraction Buffer (UNEX, Hill *et al.* 2015) (9). We extracted nucleic acids from each aliquot on the day of sample preparation (i.e., Day 0), as well as Day 14, 28, and 56. We observed that our recovery of nucleic acids from *Ancylostoma caninum* decreased by 0.0331 log₁₀ per day in ZnPVA at ambient conditions and by 0.0147 log₁₀ per day in ZnPVA at 4°C. The best-performing preservation buffer was NAP.