2023

# Use of eDNA to determine the Presence of Cryptosporidium spp. in freshwater sources in Harlan, Kentucky

Madison Hall
madison.hall@lmunet.edu

# *Use of eDNA to determine the Presence of* **Cryptosporidium** *spp. in freshwater sources in Harlan, Kentucky.*

Honors Thesis

Bachelor of Science – Biology Pre-Health

LMU Honors Program

Lincoln Memorial University, Harrogate, TN

Madison Hall

December 2023

## Abstract

*Cryptosporidium* spp. oocysts are transmitted via a fecal-oral route, e.g., water sources, and the parasite is a leading cause of waterborne disease in the United States. Research into Cryptosporidiosis in rural areas is lacking. This study utilized eDNA methods to determine if *Cryptosporidium* spp. was present in Harlan, Kentucky, an underserved area in Appalachia. We used a pump-powered commercially available Smith-Root Citizen Scientist eDNA (Vancouver, WA) system to filter freshwater sources and collect eDNA. Samples were collected from tap and spring water as controls and three times from five locations on the three tributaries of the Cumberland River: Martins Fork River, Poor Fork River, and Clover Fork River. DNA was extracted from the filter paper and a genus specific *Cryptosporidium* PCR was utilized to determine the prevalence of *Cryptosporidium*. Seventy-seven percent (n=43) of samples were PCR-positive and negative controls did not amplify. Sanger sequencing is ongoing to confirm the PCR findings and determine *Cryptosporidium* species. These data may be informative for public health in Appalachia by identifying potential risks along accessible waterways.

## Introduction

*Cryptosporidium* are intracellular protozoal parasites that belong to the phylum Apicomplexa (Class Conoidasida, Order Eucoccidiorida). Though there are many species of protists, there are difficulties in parasite identification as each species' oocyst stage is very similar (Xiao, et al., 2004). This parasite genus infects a host of vertebrates, including humans, specifically *Cryptosporidium parvum*, which causes the disease Cryptosporidiosis. Usually, this disease causes limited morbidity in healthy individuals but can cause morbidity and mortality in those with autoimmune issues (such as AIDS) or are undergoing chemotherapy (CDC,2021).

*Cryptosporidium* spp. infection can cause issues for both human and animal health. Onset of the disease varies from four to ten days with symptoms of nausea, vomiting, and diarrhea. This parasite is responsible for nearly two million annual deaths in developing countries (Boughattas et al., 2019). Incidence of cryptosporidiosis, the disease caused by *Cryptosporidium* spp., has increased in the United States (US) by 47.2% in the last decade and 241% since 2004 (CDC, 2021). This increase in cryptosporidiosis may be due to increased testing and mandatory reporting by local or state health departments; however, the increase may be due to other causes as well (CDC, 2021).

While the US has many public water systems throughout the country, it is important to maintain the safety of these systems to ensure public health. Of the roughly 156,000 public water systems, 78% are supplied by groundwater (CDC, 2021; EPA, n.d.). In the US state of Kentucky, groundwater systems can be contaminated due to poor well construction and deterioration of well conditions (Kentucky Geological Survey,

n.d). These factors may increase the risk of *Cryptosporidium* spp. in public water systems. *Cryptosporidium* spp. presence in water is difficult to determine due to low numbers and the complexity of the water matrix, so the use of additional testing methods, such as eDNA screening can be beneficial (Hassan et al., 2020).

Despite known symptoms and locations of *Cryptosporidium* spp., it is important to identify novel causes, cases, and hotspots for the pathogen. *Cryptosporidium* contamination occurred in the Appalachia region of the US during the 1990s due to contamination from cattle farms and failure of removal of protists from the water supply via Karst filters (Pasquarell, et al., 1995). Runoff from cattle farms can affect surface water quality as well and can lead to an increase in fecal coliform bacteria as well as *C. parvum* as cattle can serve as reservoirs (Pasquarell, et al., 1995). Drinking water contamination is possible and a leading cause of a *Cryptosporidium* outbreak in the United States resulted from ingesting contaminated ground water (D'Antonio et al.,1985)

Environmental DNA, or eDNA, is organismal DNA that can be found in the environment (United States Geological Survey, 2018). Environmental DNA can be used to identify species in bodies of water. The eDNA in these water bodies originates from cellular material that is shed by organisms via excrement, skin, etc. The use of eDNA is useful due to its cost-effective strategies and ability to detect rare species (Pilliod, et al., 2013). eDNA techniques have commonly been used to identify human pathogens such as *Listeria* and have proven useful when identifying fungi or pathogens in aquatic ecosystems (Papić et al. 2019). Due to the recent use eDNA techniques, few studies have been published on identifying environmental *Cryptosporidium* via eDNA

techniques. However, there is many studies have demonstrated the reliability of eDNA techniques to determine the presence of pathogens such as parasites or fungus in the water matrix. A controlled study conducted by Sieber et al. (2020) on *Batrachochytrium dendrobatidis* and *Tetracapsuloides bryosalmonae* determined that the eDNA based detection methods to identify parasites was a valid tool for field samples. Furthermore, the study highlighted that repeated collections of samples were needed.

White-tailed deer (*Odocoileus virginianus*; WTD) can serve as reservoirs for the protist and cause mass spread of the parasite. Recent studies found *C. parvum* in WTD populations, similar to cattle, suggesting that they play a role in water quality (Wells, et al., 2015). Given the wildlife population in Kentucky, especially that of WTD a potential reservoir, *Cryptosporidium* could be easily spread to waterways (Wells, et al., 2015).

In this study, I collected water samples from Harlan County, Kentucky to determine the prevalence of *Cryptosporidium* via eDNA techniques. Harlan contains many aquatic recreation areas and screening the rivers that feed these areas would prove beneficial in the safety of both persons and animals who access the site. Information on *Cryptosporidium* spp. location and prevalence could aid in ensuring the public health of those who access water sources within the area. I hypothesized that many freshwater environments would be contaminated with *Cryptosporidium* spp. due to a lack of infrastructure within Harlan, Kentucky.

## Materials and Methods

*Sample Collection*

Harlan, Kentucky, a rural county of Southeastern Kentucky, is part of the watershed of the Cumberland River—which begins in Letcher County, KY, and terminates 688 miles later in the Ohio River in Smithland, KY (Fig. 1). In Harlan, samples were collected from tributaries of the Cumberland River: the Clover Fork, Martins Fork, and Poor Fork located in Evarts, Cawood, and Baxter, Kentucky, respectively (Fig. 2). Along these rivers, five sites were tested per river among three weekly intervals for a total of 15 samples per tributary. Samples were also included from spring water sources located in the Evarts community of Harlan. Tap water samples served as a negative control for the study (Table 1). Using a Smith-Root Citizen Scientist eDNA (Vancouver, WA) system primer pump, (Fig. 3) water was collected according to manufacturer's protocol and filtered through the system. eDNA was captured on the filter paper within the pump. After the pump's completion, the filters were stored at room temperature in sealed sterile pouches according to the manufacturer's instructions until processing.

*DNA Extraction and PCR*

Each filter paper was removed with sterile forceps folded into a microcentrifuge tube. DNA from the filter paper was extracted with a Qiagen DNeasy kit, following the manufacturer's protocol for tissue extraction (Venlo, Netherlands). A PCR was used with *Cryptosporidium* spp. primers (Cry18S-S2, 5' GGTGACTCATAATAACTTTACGG 3' and Cry18S-As2, 5'ACGCTATTGGAGCTGGAATTAC 3') to determine prevalence

(Kuzehkanan et al., 2011). Gel electrophoresis was performed (Fig. 4). The positive control for the sample was synthetic *Cryptosporidium* DNA. Negative controls consisted of both tap and spring water samples in addition to extraction negative controls and PCR negative controls. DNA extraction, PCR and gel electrophoresis were conducted in separate dedicated areas with Sanger sequencing will be conducted on PCR-positive amplicons to determine *Cryptosporidum* species were present or to identify if similar Apicomplexan microorganisms were causing a false positive.

**Results**

Of the samples, 33 of the 43 (77%) samples collected had positive amplicons, with the Poor Fork tributary with the highest prevalence at 96% (Fig. 5).

**Discussion**

Our data indicated that every tributary sampled from the Cumberland River likely has *Cryptosporidium* spp. with 77% of samples (n=43) having positive amplicons. Our results indicated that there is a potentially high prevalence of *Cryptosporidium* or a related genus. Of the samples provided, which represented three tributaries of the Cumberland River (Clover Fork, Martins Fork, and Poor Fork), positives were shown for each tributary. These results supported our hypothesis and proved that the parasite is in freshwater systems in the Harlan community and serves as a public health risk for citizens of the county.

The illuminated bands did not accurately match base pair units with the ladder, indicating additional species of *Cryptosporidium* in the water samples or related species

to *Cryptosporidium* such as *Giardia*. Varying species of the parasite can display similarities on a molecular level which would bind to the designated primer and create false positives. Given the possibility of such contamination, positive PCR samples will be sequenced.

Similar to results of other studies (Wells, et. Al, 2015) a high presence of *Cryptosporidium* was found in water samples with 57% of samples (n=40) showing positive. Initial PCR results were also sent for Sanger sequencing (Wells, et al, 20115) to identify the species of this pathogen as will be done for our own results.

While this study has provided valuable insights into the presence of *Cryptosporidium* in freshwater systems in Harlan, KY, there are several areas that can be further investigated to enhance our understanding of public safety. Research into source tracking can be further investigated to determine if there are potential hotspots for the pathogen. It may also be important to research what factors are contributing to these contaminations and if health-related approaches need to be implemented in the area such as run off from cattle farms or contamination from White-tailed deer (*Odocoileus virginianus*; WTD) populations within Harlan, KY. Evaluating the public health implications of Crypto exposure on human health and infection rates among Harlan Countians would also be important as it can provide data on how dangerous this pathogen is to the health and safety of the community.

Areas of concern regarding our research can be related to the issues of detection of our pathogen. Cryptosporidium as a small oocyst is difficult to detect in the water matrix. Like other human pathogens, *Cryptosporidium* levels can fluctuate and be increasingly difficult to detect if numbers are low due to the infancy of eDNA techniques

in this field. Water contamination could also pose issues to this project as it would make detection for the desired pathogen difficult if a related species is also present in the water samples collected.

Other research areas could be performed concerning water treatment and filtration methods to ensure the *Cryptosporidium* is being removed from recreational sources, such as splash pads, and household water supplies. Further research investigating potential reservoirs for this pathogen, such as white-tail deer or black bear, would also prove beneficial as it would identify an additional source of contamination. Studies could also be conducted on sources of drinking water such as springs to ensure that the protist is not located in any consumable water sources to avoid potential outbreaks in the community.

Finally, collaborative efforts should be conducted to provide community education and outreach for citizens of the county on water hygiene and awareness of the protist and the severity of becoming infected with it.

The overall purpose of this research is to identify the impact *Cryptosporidium* has on public health within Harlan County, KY. With little public knowledge of this pathogen in the region, Harlan Countians may become infected with the pathogen and not seek medical treatment which may prove lethal in some cases. In addition to the impact this pathogen has on the Harlan region, the contamination of this pathogen into other bodies of water can further expand the risk of public illness to other environments.

**Acknowledgements**

I would like to acknowledge the faculty, departments, and organizations who contributed to this project. Dr. Barbara C. Shock, who is my mentor, has guided me throughout the entirety of this project and has helped me tremendously. I would also like to thank Dr. Kistler and my fellow classmate Logan Taylor for their assistance in the lab during the DNA extraction process. I also would like to acknowledge the Appalachian College Association for providing the grant needed to conduct this research via the Ledford Scholarship. Finally, thank you to the Lincoln Memorial University School of Mathematics and Sciences for providing the facilities and tools needed to conduct the lab portion of this work.

**References**

Boughattas, S., Behnke, J. M., Al-Sadeq, D., Ismail, A., & Abu-Madi, M. (2019). Cryptosporidium spp., prevalence, molecular characterisation and socio-demographic risk factors among immigrants in Qatar. *PLOS Neglected Tropical Diseases, 13*(10), e0007750. https://doi.org/10.1371/journal.pntd.0007750

Boyer, D. G., & Kuczynska, E. (2003). Storm and seasonal distributions of fecal coliforms and Cryptosporidium in a spring1. *JAWRA Journal of the American Water Resources Association, 39*(6), 1449–1456. https://doi.org/10.1111/j.1752-1688.2003.tb04430.x

Centers for Disease Control and Prevention. (2021, November 24). Illness & symptoms. Centers for Disease Control and Prevention. https://www.cdc.gov/parasites/crypto/illness.html

Centers for Disease Control and Prevention. (2021, September 15). Cryptosporidiosis NNDSS Summary Report for 2019. https://www.cdc.gov/healthywater/surveillance/cryptosporidium/cryptosporidium-2019.html

D'Antonio, R. G. (1985). A waterborne outbreak of cryptosporidiosis in normal hosts. *Annals of Internal Medicine, 103*(6_Part_1), 886. https://doi.org/10.7326/0003-4819-103-6-886

Daniel Boone National Forest - Cumberland River - London. (n.d.). Forest Service National Website. https://www.fs.usda.gov/recarea/dbnf/recarea/?recid=72125

(2007). Factoids: Drinking Water and Ground Water Statistics for 2007... - EPA. https://nepis.epa.gov/Exe/ZyPDF.cgi/P100N2VG.PDF?Dockey=P100N2VG.PDF

Frequently asked questions about groundwater and water wells in Kentucky. (n.d.). https://www.uky.edu/KGS/water/general/wfaq.htm

Gerace, E., Di Presti, V., & Biondo, C. (2019). Cryptosporidium infection: Epidemiology, pathogenesis, and differential diagnosis. *European Journal of Microbiology and Immunology, 9*(4), 119–123. https://doi.org/10.1556/1886.2019.00019

Hassan, E. M., Örmeci, B., DeRosa, M. C., Dixon, B. R., Sattar, S. A., & Iqbal, A. (2020). A review of Cryptosporidium spp. and their detection in water. *Water Science and Technology, 83*(1), 1–25. https://doi.org/10.2166/wst.2020.515

Kuzehkanan, B., A., Rezaeian, M., Zeraati, H., Mohebali, M., Meamar, A. R., Babaei, Z., Kashi, L., Heydarnezhadi, M., & Rezaie, S. (2011). A Sensitive and Specific PCR Based Method for Identification of Cryptosporidium Sp. Using New Primers from 18S Ribosomal RNA. *Iranian journal of parasitology*, *6*(4), 1–7.

Papić, B., Golob, M., Kušar, D., Pate, M., & Zdovc, I. (2019). Source tracking on a dairy farm reveals a high occurrence of subclinical mastitis due to hypervirulent Listeria monocytogenes clonal complexes. *Journal of Applied Microbiology, 127*(5), 1349–1361. https://doi.org/10.1111/jam.14418

Pasquarell, G. C., & Boyer, D. G. (1995). Agricultural impacts on bacterial water quality in karst groundwater. *Journal of Environmental Quality, 24*(5), 959–969. https://doi.org/10.2134/jeq1995.00472425002400050026x

Pilliod, D. S., Goldberg, C. S., Laramie, M. B., & Waits, L. P. (2013). Application of environmental DNA for inventory and monitoring of aquatic species. *U.S. Geological Survey Fact Sheet 2012-3146*, 4 p.

Public Water Systems. (2021, March 30). Centers for Disease Control and Prevention. https://www.cdc.gov/healthywater/drinking/public/index.html

Ryan, U., & Hijjawi, N. (2015). New developments in Cryptosporidium research. *International Journal for Parasitology, 45*(6), 367–373. https://doi.org/10.1016/j.ijpara.2015.01.009

Sieber, N., Hartikainen, H., & Vorburger, C. (2020). Validation of an Edna-based method for the detection of wildlife pathogens in water. *Diseases of Aquatic Organisms, 141*, 171–184. https://doi.org/10.3354/dao03524

Water Science School | U.S. Geological Survey. (n.d.). https://www.usgs.gov/special-topics/water-science-school

Wells, B., Shaw, H., Hotchkiss, E., Gilray, J., Ayton, R., Green, J., Katzer, F., Wells, A., & Innes, E. (2015). Prevalence, species identification and genotyping Cryptosporidium from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. *Parasites & Vectors, 8*(1), 66. https://doi.org/10.1186/s13071-015-0684-x

Xiao, L., Fayer, R., Ryan, U., & Upton, S. J. (2004). Cryptosporidium taxonomy: Recent advances and implications for public health. *Clinical Microbiology Reviews, 17*(1), 72–97. https://doi.org/10.1128/cmr.17.1.72-97.2004

**Figures and Tables**

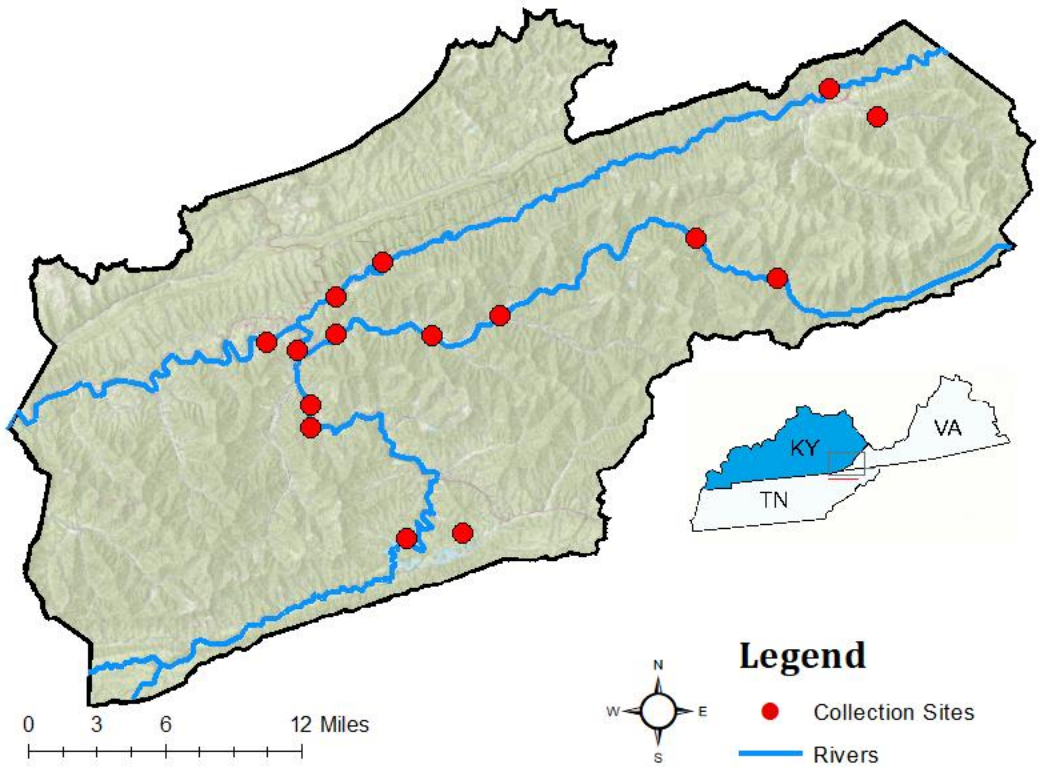


**Figure 1**: eDNA sample collection sites on the Clover Fork, Poor Fork, and Martin Fork tributaries of the Cumberland River in Harlan County, KY

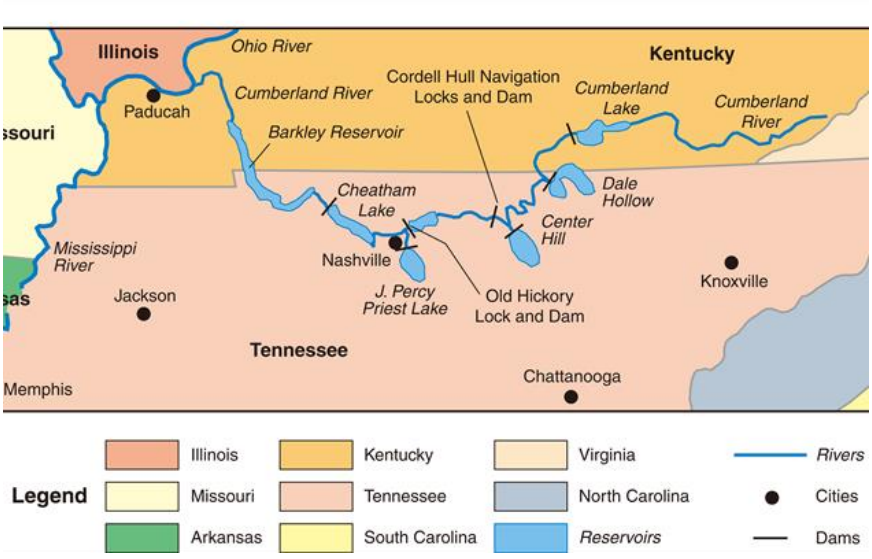


**Figure 2**: Map showcasing the watershed of Cumberland River through Kentucky and Tennessee. Source: https://www.scirp.org/journal/paperinformation.aspx?paperid=107197

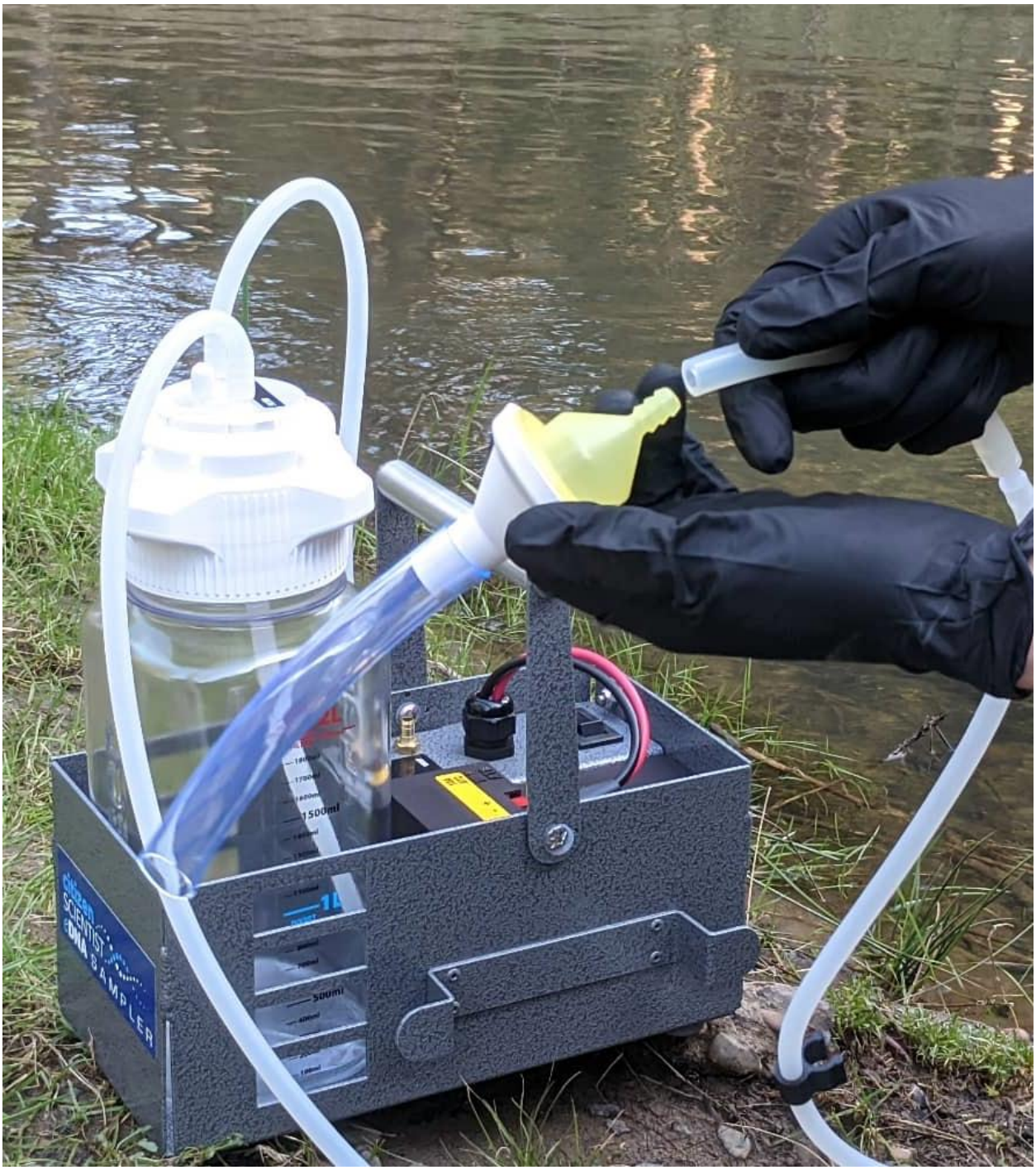

**Figure** 3: Image of the Citizen Scientist™ primer pump.

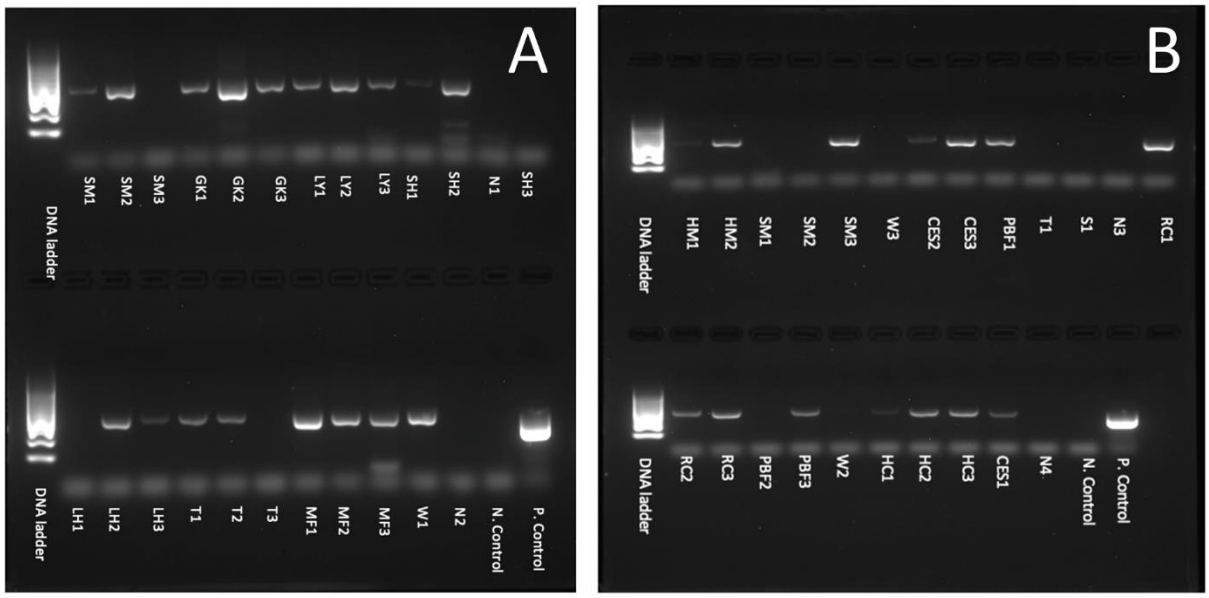


**Figure 4:** Gel electrophoresis of *Cryptosporidium* spp. PCR of eDNA samples collected from Harlan County, KY. Samples were collected from three tributaries of the Cumberland River. “N. Control” denotes PCR negative control. “P. Control” denotes PCR positive control. N1 to N4 denote DNA extraction negative controls. See Table 1 for sample abbreviation locations.

**Table 1.** eDNA collection sites and number of samples per location on three tributaries of the Cumberland River. Abbreviations correlate to sample abbreviations on figures. Additionally one sample each were collected from spring water and tap water.

| Clover fork (n=14) | Poor fork (n=15) | Martins fork (n=12) |
|---|---|---|
| **LH (n=3)**<br>**Logan's House** | HC (n=3)<br>Harlan County High School | MF (n=3)<br>Martins Fork Trout Release |
| **SWM (n=3)**<br>**Saw Mill** | PBF (n=3)<br>Cumberland Little League | GK (n=3)<br>Grays Knob |
| **SH (n=3)**<br>**Slope Hollow** | LY (n=3)<br>Loyall | W (n=3)<br>Walmart |
| **T (n=3)**<br>**Tunnels Ivy Hill** | CES (n=3)<br>Cumberland Elementary | SM (n=3)<br>Stone Mountain |
| **HM (n=2)**<br>**Holmes Mill** | RC (n=3)<br>Rosspoint Church | |

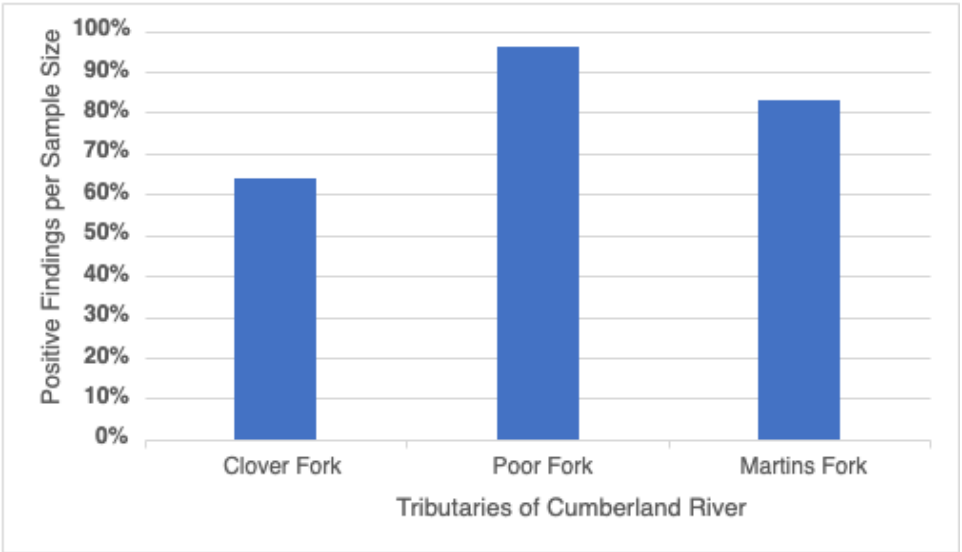


**Figure 5**: *Cryptosporidium* PCR positive percentages for eDNA sample collections (n=43) on three tributaries of the Cumberland River: Clover Fork (14 samples), Poor Fork (15 samples), Martin's Fork (12 samples).