Methods and Applications

Development of a High-Throughput qPCR Assay for Detecting Waterborne Protozoa and Helminths Across Different Environmental Media in China

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ABSTRACT

Introduction: The establishment of a high-throughput quantification approach for waterborne pathogenic protozoa and helminths is crucial for rapid screening and health risk assessment.

Methods: We developed a high-throughput quantitative polymerase chain reaction (HT-qPCR) assay targeting 19 waterborne protozoa and 3 waterborne helminths and validated its sensitivity, specificity, and repeatability. The assay was then applied to test various environmental media samples.

Results: The HT-qPCR assay's limit of detection (LOD) was 5×10^2 copies/μL DNA, and its specificity was confirmed using *Giardia* and *Cryptosporidium* standards. Repeatability, assessed through intra- and inter-group experiments, yielded a coefficient of variation (*CV*) of 1.0%–4.6% and 1.2%–6.4% at concentrations of 1×10^5 and 1×10^4 copies/μL, respectively. The R^2 values of the 22 standard curves ranged from 0.983 to 0.998, with amplification efficiencies between 80% and 107%. In drinking water sources, sludge from municipal wastewater treatment plants (MWTPs), and livestock manure samples, 17 of 22 targets were detected, with *Acanthamoeba* genus (50.0%), *Acanthamoeba castellanii* (11.8%), and *Enterocytozoon bieneusi* (11.8%) showing high prevalence. *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Cyclospora cayetanensis* were simultaneously found in all three sample types.

Discussion: This study presents a useful tool for the rapid detection of waterborne protozoa and helminths in complex environmental microbiomes, providing scientific data for monitoring cross-media transmission and controlling microbial risk from a One Health perspective.

Water is a common transmission vector for numerous pathogens. Many waterborne protozoa and helminths are zoonotic, posing significant risks to both humans and animals, particularly through the ingestion of fecally contaminated water. Even at relatively low environmental concentrations, these pathogens can present substantial public health risks, with vulnerable populations such as the elderly, children, and individuals with acquired immunodeficiency syndrome being particularly susceptible (*1*). A notable example is the 1993 cryptosporidiosis outbreak in Milwaukee, United States, which resulted in approximately 400,000 infections (*2*). From a One Health perspective, cross-media monitoring and understanding transmission mechanisms are crucial for controlling waterborne protozoa and helminths in the environment-animal-human continuum.

Among waterborne protozoa and helminths, *Cryptosporidium* and *Giardia* are included in the National Standard for Drinking Water Quality of China (GB 5749-2022), the European Union's drinking water quality standards, and the World Health Organization's (WHO) Guidelines for drinking-water quality. They are also classified as Class B pathogenic microorganisms by the National Institutes of Health, USA (*3–6*). No other waterborne protozoa are included in these standards and guidelines. In addition to *Cryptosporidium* and *Giardia*, other pathogens such as *Echinococcus ameba*, *Microsporidium*, *Enterocytozoon bieneusi*, *Toxoplasma gondii*, and *Schistosoma* have also been linked to waterborne diseases in both humans and animals (*1,7*). Therefore, there is an urgent need for an efficient detection method that can cover a broad spectrum of these waterborne protozoa and helminths. Traditional detection methods for pathogenic protozoa and helminths mainly include microscopy, quantitative polymerase chain reaction (qPCR), nested PCR, and loop-mediated isothermal amplification (LAMP) (*8*). Recent advancements in high-throughput technologies

have enabled the detection of microorganisms within complex microbiomes. High-throughput qPCR (HT-qPCR) has been widely employed for monitoring of bacterial pathogens and viruses in environmental samples (9–11). However, a high-throughput approach specifically targeting waterborne protozoa and helminths is still lacking, impeding the rapid screening of these pathogens in the environment. In this study, we developed and validated an HT-qPCR method for the detection of protozoa and helminths and applied it to screen for waterborne pathogens in environmental samples and related pollution sources from a One-Health perspective.

METHODS

Sampling Based on a One Health Perspective

A One Health perspective emphasizes the close links between human, animal, and environmental health. This study selected environmental samples, including: 1) drinking water source samples from the Yangtze River in August 2023 (n=23) and Yellow River in October 2023 and January 2024 (n=23) in China (n=46); 2) sludge samples from municipal wastewater treatment plants (MWTPs) in 2015 (n=12); and 3) livestock manure in 2020 and 2021 from pigs (n=6), chickens (n=2), sheep (n=1), and cattle (n=1). These samples are closely related to human, animal, and environmental health, respectively, and were used to investigate the potential risks of pathogenic microorganisms. Studying and managing these samples will help us better understand and respond to various health risks and achieve common health goals for humans, animals, and the environment. For drinking water source samples, 10 L of water was concentrated using the calcium carbonate flocculation method, and the concentrate was transferred for DNA extraction. Sludge and livestock manure samples were used directly for DNA extraction. Environmental DNA was extracted from 0.5 g of sample concentrate using the FastDNA SPIN Kit for Soil and eluted in 75 μL of DES. Extracted DNA was stored at –20 °C until use.

High-throughput qPCR Setup

Twenty-two pathogenic taxa, including 19 protozoa and 3 helminths, were selected as detection targets (Table 1). These taxa were chosen based on their waterborne characteristics and potential environmental dissemination risk as outlined in the WHO Water

Quality Guidelines (4th edition) (3). Additionally, 18S rRNA and 16S rRNA genes served as quality controls. DNA was diluted to approximately 20 ng/μL, and 400 ng/μL bovine serum albumin (BSA) was added to each qPCR system to improve amplification efficiency and mitigate potential inhibition. The amplification efficiency of all reactions was between 80% and 120%.

The PCR primers and probes were designed using a Thermo Fisher proprietary process, with T_m values between 58 °C and 62 °C (12). Primer lengths were between 9 bp and 40 bp, with GC content ranging from 30% to 80%. Primer and probe information, including assay IDs, is shown in Supplementary Table S1 and stored in the Odyssey data by Thermo Fisher. The HT-qPCR assay for detecting waterborne protozoa and helminths was conducted on OpenArray chips using an Applied Biosystems QuantStudio 12K Flex instrument (Thermo Fisher, USA). The OpenArray Module enables simultaneous operation of up to four OpenArray chips, allowing testing of up to 192 samples over a 3-hour period, significantly increasing reaction throughput.

Validation and Application of the HT-qPCR Assay

For the sensitivity test, the standard plasmid (1×10^7 copies/μL) was diluted to a gradient of 1×10^6, 1×10^5, 1×10^4, 1×10^3, 5×10^2, 4×10^2, 3×10^2, 2×10^2, and 1×10^2 copies/μL (3 replicates of each sample), and HT-qPCR assays were performed. The minimum concentration of gene copies from the standard series was considered the qPCR limit of detection (LOD). The unit of LOD was gene copies per microliter of DNA (13). For the specificity test, quality control standards of *Giardia lamblia* and *Cryptosporidium parvum* (Waterborne™, USA) were used for the qPCR assay. For the repeatability test, plasmid standards of 1×10^5 and 1×10^4 copies/μL were used for intra- and inter-group experiments, and the average Cycle threshold (Ct) value and coefficient of variation (CV) were calculated.

The standard plasmid (1×10^7 copies/μL) was diluted to six gradient concentrations of 1×10^6, 1×10^5, 1×10^4, 1×10^3, 5×10^2, and 1×10^2 copies/μL, with three replicates per concentration. RNase-free ddH$_2$O served as a negative control. The kinetic amplification curve and PCR amplification cycle threshold (Ct value) were obtained, and a standard curve was constructed by plotting the Ct value (vertical axis) against the log$_{10}$ (plasmid copy number) (horizontal axis). Method

TABLE 1. Basic information of waterborne protozoa and helminths.

Pathogen	Detection target	Health significance	Persistence in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Acanthamoeba	*Acanthamoeba*	−	−	−	−	−
	Acanthamoeba culbertsoni	High	May multiply	Low	High	No
	Acanthamoeba castellanii	High	May multiply	Low	High	No
	Acanthamoeba polyphaga	High	May multiply	Low	High	No
Balantioides	*Balantidium coli*	High	Moderate	High	High	No
Blastocystis	*Blastocystis hominis*	High	May multiply	High	Moderate	Yes
Cryptosporidium	*Cryptosporidium* spp.	−	−	−	−	−
	Cryptosporidium parvum	High	Long	High	High	Yes
	Cryptosporidium hominis	High	Long	High	High	Yes
	Cryptosporidium meleagridis	High	Long	High	High	Yes
Cyclospora	*Cyclospora cayetanensis*	High	Long	High	High	Yes
Enterocytozoon	*Enterocytozoon bieneusi*	High	Long	High	High	Yes
Entamoeba	*Entamoeba histolytica*	High	Moderate	High	High	No
Encephalitozoon	*Encephalitozoon*	High	Moderate	High	Moderate	Yes
Giardia spp.	*Giardia* spp.	−	−	−	−	−
	Giardia lamblia	High	Moderate	High	High	Yes
Naegleria	*Naegleria fowleri*	High	May multiply	Low	Moderate	No
Isospora	*Isospora belli*	Moderate	Moderate	High	High	Yes
Toxoplasma gondii	*Toxoplasma gondii*	High	Long	High	High	Yes
Echinococcus	*Echinococcus*	High	Long	Low	Moderate	Yes
Dracunculus	*Dracunculus medinensis*	Low	Moderate	Moderate	High	No
Schistosoma	*Schistosoma* spp.	High	Short	Moderate	High	Yes

Note: "−"=not available.

validity was verified based on the correlation coefficients, slopes, and amplification efficiencies of the standard curves for each detection target. Following development, the HT-qPCR assay was used to detect waterborne protozoa and helminths in various environmental samples.

RESULTS

Establishment and Validation of HT-qPCR Assay

Table 2 outlines the sensitivity of the HT-qPCR assay, evaluated using a gradient of positive plasmid concentrations. The results showed that all 24 detection targets were reliably detected at positive plasmid concentrations exceeding 5×10^2 copies/μL DNA. At concentrations of 4×10^2 and 3×10^2 copies/μL DNA, 15 and 12 targets were stably detected, respectively. When the concentration dropped below 2×10^2 copies/μL DNA, none of the 24

targets were consistently detected. Thus, the limit of detection for the HT-qPCR assay was established at 5×10^2 copies/μL DNA.

The specificity of the method was verified using standard samples of *Cryptosporidium parvum* and *Giardia lamblia*, alongside a negative control of RNase-free water. For *Cryptosporidium parvum*, only the qPCR assays for *Cryptosporidium* spp. and *Cryptosporidium parvum* produced positive results with Ct values less than 35 in the amplification curves, while no amplification was observed in the other assays. Similarly, for *Giardia lamblia*, only the qPCR assays targeting *Giardia* spp. and *Giardia lamblia* showed positive results with Ct values below 35. No positive results were observed for any of the 24 detection targets in the negative control. To assess the repeatability of the method, intra- and inter-group experiments were performed using positive plasmid concentrations of 10^5 and 10^4 copies/μL DNA. When the plasmid concentration was 10^5 copies/μL DNA, the *CV* for intra- and inter-group experiments ranged

TABLE 2. Sensitivity test results of the HT−qPCR assay.

Positive plasmid copy number (copies/µL)	Ct value within group			Note
	Average value	Standard deviation	Coefficient of variation (%)	
1×10^6	17.4–19.3	0.08–0.4	0.4–2.2	Stable detection of 24 targets
1×10^5	20.8–23.0	0.2–0.9	1.0–4.1	Stable detection of 24 targets
1×10^4	25.0–27.3	0.4–1.2	1.7–4.6	Stable detection of 24 targets
1×10^3	27.9–30.7	0.5–2.6	1.5–8.6	Stable detection of 24 targets
5×10^2	28.8–31.4	0.03–1.0	0.1–3.3	Stable detection of 24 targets
4×10^2	ND–33.5	0.6–9.3	1.9–50.7	Stable detection of 15 out of 24 targets
3×10^2	ND–32.3	0.4–8.6	1.2–37.6	Stable detection of 12 out of 24 targets
2×10^2	ND–32.5	0.2–9.6	0.5–46.4	All 24 target detections are unstable
1×10^2	ND–32.9	0.2–7.2	0.9–48.5	All 24 target detections are unstable

Abbreviation: HT-qPCR=high-throughput quantitative polymerase chain reaction; Ct=Cycle threshold.

from 1.0% to 4.1% and 1.2% to 5.5%, respectively. At 10^4 copies/µL DNA, the *CV* range for intra- and inter-group experiments was 1.7%–4.6% and 3.0%–6.4%, respectively. A *CV* of less than 10% confirmed the method's acceptable repeatability.

A total of 24 standard curves for 19 protozoa, 3 helminths, and 18S rRNA and 16S rRNA genes were generated (Supplementary Table S1, available at https://weekly.chinacdc.cn/). The correlation coefficients (R^2) ranged from 0.983 to 0.998, with amplification efficiencies between 80% and 107%, indicating reliable stability across all detected targets under the experimental conditions.

Application to Diverse Environmental Samples

Table 3 presents the diagnostic performance of the HT-qPCR assay for detecting protozoa and helminths in environmental samples from drinking water sources (*n*=46), sludge from MWTPs (*n*=12), and livestock manure (*n*=10). In total, 17 of 22 targets were detected across all samples, with the three most prevalent being the *Acanthamoeba* genus (50.0%), *Acanthamoeba castellanii* (11.8%), and *Enterocytozoon bieneusi* (11.8%). The distribution of protozoa and helminths varied among the three media types. Thirteen were detected in drinking water sources, 13 in MWTP sludge, and 11 in livestock manure.

In drinking water sources, the frequently detected targets were *Acanthamoeba* genus, *Naegleria fowleri*, and *Acanthamoeba castellanii*, with concentrations ranging from n.d.–5.0×10^5, n.d.–1.5×10^5, and n.d.–4.7×10^5 gene copies/L. In MWTP sludge, the dominant targets were *Acanthamoeba* genus, *Isospora belli*, and *Acanthamoeba polyphaga*, with concentrations

of n.d.–7.2×10^5, n.d.–1.2×10^5, and n.d.–1.1×10^5 gene copies/g, respectively. In livestock manure, *Balantidium coli*, *Cyclospora cayetanensis*, and *Enterocytozoon bieneusi* were detected most frequently, with concentrations of n.d.–9.9×10^5, n.d.–4.7×10^4, and n.d.–1.0×10^4 gene copies/g, respectively (Supplementary Table S2, available at https://weekly.chinacdc.cn/). Notably, *Cryptosporidium* was detected more frequently than *Giardia*.

Importantly, *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Cyclospora cayetanensis*, *Balantidium coli*, and *Isospora belli* were simultaneously detected in drinking water sources, MWTP sludge, and livestock manure.

DISCUSSION

This study developed and validated an HT-qPCR assay capable of detecting 22 waterborne protozoa and helminths. This assay addresses the limitations of low throughput in existing diagnostic approaches while improving accuracy and providing a more cost-effective solution for assessing the risks of protozoa and helminths from a One Health perspective.

Compared to current detection methods, this approach significantly enhances detection efficiency. Using four OpenArray chips in a single run on the Applied Biosystems QuantStudio 12K Flex instrument, 22 targets across up to 192 samples can be analyzed within 3 hours. This rapid data generation is advantageous for large-scale monitoring and initial screening, facilitating subsequent risk assessments.

Infectious diseases caused by pathogenic bacteria, viruses, and parasites (e.g., protozoa and helminths) are the most prevalent health risks linked to drinking water. The primary threat to public health from

TABLE 3. Protozoa and helminth DR in environmental samples by HT-qPCR.

Type	No.	Target	Drinking water sources (DR%)	MWTP sludge (DR%)	Livestock manure (DR%)
Protozoa	1	*Acanthamoeba* genus	54.3	58.0	20.0
	2	*Acanthamoeba culbertsoni*	4.3	8.3	10.0
	3	*Acanthamoeba castellanii*	10.9	8.3	20.0
	4	*Acanthamoeba polyphaga*	2.2	25.0	−
	5	*Balantidium coli*	2.2	8.3	40.0
	6	*Blastocystis hominis*	−	−	10.0
	7	*Cryptosporidium* spp.	8.7	16.7	10.0
	8	*Cryptosporidium meleagridis*	−	−	−
	9	*Cryptosporidium hominis*	−	−	−
	10	*Cryptosporidium parvum*	−	8.3	−
	11	*Cyclospora cayetanensis*	4.3	8.3	20.0
	12	*Isospora belli*	2.2	33.3	10.0
	13	*Encephalitozoon*	2.2	−	−
	14	*Entamoeba histolytica*	−	8.3	−
	15	*Enterocytozoon bieneusi*	8.7	16.7	20.0
	16	*Giardia* spp.	−	−	−
	17	*Giardia lamblia*		16.7	−
	18	*Naegleria fowleri*	13.1	−	−
	19	*Toxoplasma gondii*	−	−	10.0
Helminths	20	*Echinococcus*	−	−	−
	21	*Dracunculus medinensis*	2.2	8.3	10.0
	22	*Schistosoma* spp.	−	−	−

Note: "−"=not detectable.
Abbreviation: MWTP=municipal wastewater treatment plant; DR=detection rates; HT-qPCR=high-throughput quantitative polymerase chain reaction.

waterborne microbes stems from drinking water contaminated with human and animal excreta (*3*). In this study, several parasites, including *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Cyclospora cayetanensis*, were simultaneously detected in drinking water sources, MWTP sludge, and livestock manure. Animal breeding near the drinking water sources may be a potential source of contamination (*14–15*). For instance, waterborne transmission of *Enterocytozoon bieneusi* was reported during the 2013 "Pig Carcass Disposal Incident" in Shanghai, China, where dead pigs in the Huangpu River contributed to *Enterocytozoon bieneusi* contamination with animal and human genotypes, posing a threat to drinking water safety (*16*). The widespread existence of zoonotic *E. bieneusi* genotypes (D, EbpC, Type IV) in dogs and cats indicates that they are potential sources of environmental contamination and human infections (*17*). Thus, beyond *Cryptosporidium* and *Giardia*, which are already included in the National Standard for Drinking Water Quality of China (GB 5749-

2022), monitoring the cross-media transmission of a broader range of protozoa and helminths is crucial for future public health management from a One Health perspective. Considering that RNA may reflect the viable pathogens in the samples, in the future, we will simultaneously quantify DNA and RNA from protozoa and helminths to improve the identification of viable pathogens (*11*).

This study provides a valuable tool for rapid screening and source tracking of waterborne protozoa and helminths. It is primarily used for high-throughput screening to determine the prevalence of a wide range of protozoa and helminths, characterize prevalence, and provide baseline data for identifying reference pathogens. It should be noted that the current HT-qPCR method does not allow for genotype identification of the detected pathogens. While suitable for routine monitoring, further research is needed to explore the potential for animal-environment-human transmission of waterborne pathogens.

Conflicts of interest: No conflicts of interest.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Standard curves, correlation coefficients, amplification efficiencies for detection target of HT−qPCR.

Type	No.	Target	Standard curve	Correlation coefficient	Amplification efficiency (%)	Assay ID*
Protozoa	1	*Acanthamoeba*	y=−3.80x+41.12	R^2=0.997	83	AP7DU7W
	2	*Acanthamoeba culbertsoni*	y=−3.69x+41.58	R^2=0.993	87	APEPZZ9
	3	*Acanthamoeba castellanii*	y=−3.91x+41.68	R^2=0.996	80	APDJ7GC
	4	*Acanthamoeba polyphaga*	y=−3.81x+41.84	R^2=0.996	83	APAAJCZ
	5	*Balantidium coli*	y=−3.70x+40.84	R^2=0.995	86	AP2XA9F
	6	*Blastocystis hominis*	y=−3.68x+40.52	R^2=0.995	87	APGZFRJ
	7	*Cryptosporidium hominis*	y=−3.80x+41.12	R^2=0.998	83	APAAJDD
	8	*Cryptosporidium meleagridis*	y=−3.93x+41.66	R^2=0.998	80	AP9HTZD
	9	*Cryptosporidium* spp.	y=−3.72x+40.53	R^2=0.987	86	Pr07922253_s1
	10	*Cryptosporidium parvum*	y=−3.16x+37.60	R^2=0.985	107	APGZGDP
	11	*Cyclospora cayetanensis*	y=−3.47x+38.39	R^2=0.997	94	Pr07922173_s1
	12	*Encephalitozoon*	y=−3.67x+40.76	R^2=0.987	87	APDJ7HX
	13	*Entamoeba histolytica*	y=−3.69x+40.00	R^2=0.998	87	AP2XCMY
	14	*Enterocytozoon bieneusi*	y=−3.88x+41.16	R^2=0.999	81	AP7DWCR
	15	*Giardia* spp.	y=−3.74x+40.42	R^2=0.996	85	AP473C7
	16	*Giardia lamblia*	y=−3.21x+37.45	R^2=0.983	109	APZTK62/AP33ACW/ AP7DXGR
	17	*Isospora belli*	y=−3.64x+39.34	R^2=0.996	88	APNKRWX
	18	*Naegleria fowleri*	y=−3.85x+41.33	R^2=0.997	82	AP2XERK
	19	*Toxoplasma gondii*	y=−3.63x+39.23	R^2=0.998	88	APDJY3F
Helminths	20	*Dracunculus medinensis*	y=−3.94x+40.89	R^2=0.997	80	APPRVCD
	21	*Echinococcus*	y=−3.79x+40.48	R^2=0.994	84	APGZMAC
	22	*Schistosoma* spp.	y=−3.74x+38.16	R^2=0.995	85	APNKXJZ/APU69VP / APWC3FM/APXGWZJ
Quality control	23	16S rRNA gene	y=−3.20x+36.56	R^2=0.995	105	Ba04930791_s1
	24	18S rRNA gene	y=−3.71x+38.47	R^2=0.997	86	Pa99999901_s1

* The PCR primers and probes were designed by a ThermoFisher proprietary process. Primer and probe information are stored in the Odyssey data by ThermoFisher.

SUPPLEMENTARY TABLE S2. Concentration (mean value and range) of detection targets in different media.

Type	No.	Target	Drinking water sources		MWTP Sludge		Livestock manure	
			Mean value (gc/L)	Range (gc/L)	Mean value (gc/g)	Range (gc/g)	Mean value (gc/g)	Range (gc/g)
Protozoa	1	*Acanthamoeba genus*	$7.5×10^4$	nd−$5.0×10^5$	$2.3×10^5$	nd−$7.2×10^5$	$6.7×10^3$	nd−$5.3×10^4$
	2	*Acanthamoeba culbertsoni*	$3.4×10^3$	nd−$8.8×10^4$	$4.8×10^3$	nd−$5.7×10^4$	$6.0×10^2$	nd−$6.0×10^3$
	3	*Acanthamoeba castellanii*	$2.4×10^4$	nd−$4.7×10^5$	$5.2×10^3$	nd−$6.2×10^4$	$2.2×10^3$	nd−$1.7×10^4$
	4	*Acanthamoeba polyphaga*	$2.6×10^3$	nd−$1.2×10^5$	$1.5×10^4$	nd−$1.1×10^5$	nd	nd
	5	*Balantidium coli*	$2.0×10^3$	nd−$9.0×10^4$	$7.4×10^3$	nd−$8.9×10^4$	$1.0×10^5$	nd−$9.9×10^5$
	6	*Blastocystis hominis*	nd	nd	nd	nd	$2.5×10^3$	nd−$2.5×10^4$
	7	*Cryptosporidium* spp.	$3.5×10^3$	nd−$4.5×10^4$	$1.9×10^4$	nd−$1.8×10^5$	$4.1×10^2$	nd−$4.1×10^3$
	8	*Cryptosporidium meleagridis*	nd	nd	nd	nd	nd	nd
	9	*Cryptosporidium hominis*	nd	nd	nd	nd	nd	nd
	10	*Cryptosporidium parvum*	nd	nd	$6.9×10^3$	nd−$8.3×10^4$	nd	nd
	11	*Cyclospora cayetanensis*	4.3	nd−$5.7×10^4$	$1.2×10^5$	nd−$1.0×10^4$	$4.9×10^3$	nd−$4.7×10^4$
	12	*Isospora belli*	$1.3×10^3$	nd−$6.0×10^4$	$2.3×10^4$	nd−$1.2×10^5$	$6.7×10^3$	nd−$6.7×10^4$
	13	*Encephalitozoon*	$9.6×10^2$	nd−$4.4×10^4$	nd	nd	nd	nd
	14	*Entamoeba histolytica*	nd	nd	$4.5×10^3$	nd−$5.4×10^4$	nd	nd
	15	*Enterocytozoon bieneusi*	$3.5×10^3$	nd−$6.5×10^4$	$9.0×10^3$	nd−$4.9×10^4$	$1.4×10^3$	nd−$1.0×10^4$
	16	*Giardia* spp.	nd	nd	nd	nd	nd	nd
	17	*Giardia lamblia*	nd	nd	$1.8×10^4$	nd−$1.8×10^5$	nd	nd
	18	*Naegleria fowleri*	$8.9×10^3$	nd−$1.5×10^5$	nd	nd	nd	nd
	19	*Toxoplasma gondii*	nd	nd	nd	nd	$6.7×10^3$	nd−$6.7×10^4$
Helminths	20	*Echinococcus*	nd	nd	nd	nd	nd	nd
	21	*Dracunculus medinensis*	$9.0×10^2$	nd−$4.1×10^4$	$4.6×10^3$	nd−$5.6×10^4$	$3.0×10^3$	nd−$3.0×10^4$
	22	*Schistosoma* spp.	nd	nd	nd	nd	nd	nd

Abbreviation: MWTP=municipal wastewater treatment plant; nd=not detectable; gc=gene copies.