Molecular surveillance of *Cryptosporidium* spp. for microbial source tracking of fecal contamination in Laguna Lake, Philippines

Laurice Beatrice Raphaelle O. dela Peña, Mark Raymond A. Vejano and Windell L. Rivera

ABSTRACT

Water quality deterioration in source waters poses increased health, environmental, and economic risks. Here, we genotyped *Cryptosporidium* spp. obtained from water samples of Laguna Lake, Philippines, and its tributaries for the purpose of source-tracking fecal contamination. A total of 104 surface water samples were collected over a 1-year period (March 2018 to April 2019). Detection of *Cryptosporidium* was carried out using genus-specific primers targeting a fragment of the small subunit (SSU) rRNA gene. The study revealed 8 (14%) tributary samples and 1 (2.77%) lake sample positive for contamination. The species were determined to be *C. parvum* ($n = 4$), *C. muris* ($n = 2$), *C. hominis* ($n = 1$), *C. galli* ($n = 1$), *C. baileyi* ($n = 1$), *C. suis* ($n = 1$), as well as rat genotype IV ($n = 1$). Two species were detected in duck (*C. baileyi)* and cattle (*C. parvum)* fecal samples. The data presented suggest that *Cryptosporidium* contamination is likely to come from sewage or human feces as well as various agricultural sources (i.e. cattle, swine, and poultry). This information reveals the importance of mitigating fecal pollution in the lake system and minimizing health risks due to exposure to zoonotic *Cryptosporidium* species.

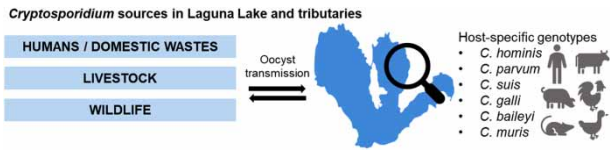
Key words | *Cryptosporidium*, Laguna Lake, microbial source tracking, Philippines, surface water

Laurice Beatrice Raphaelle O. dela Peña
Mark Raymond A. Vejano
Windell L. Rivera (corresponding author)
Pathogen-Host-Environment Interactions Research Laboratory, Institute of Biology, College of Science,
University of the Philippines Diliman,
Quezon City 1101,
Philippines
E-mail: *wlrivera@science.upd.edu.ph*

HIGHLIGHTS

- Zoonotic and pathogenic species of *Cryptosporidium* in a freshwater reservoir point to domestic and agricultural sources of fecal contamination.
- *Cryptosporidium* can be a potential marker for microbial source tracking in the Laguna Lake watershed to augment current monitoring efforts.

GRAPHICAL ABSTRACT



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INTRODUCTION

Laguna Lake (Laguna de Bay) is the largest inland lake in the Philippines. The lake services 16 million residents surrounding the area, from the neighboring provinces of Rizal, Laguna, Batangas, Quezon, and Metro Manila. The water is widely used for domestic, commercial, and industrial purposes, including aquaculture and agriculture (LLDA 2018). However, influx of high pollution loads has been reported to result in a decline of the lake water quality (Santos-Borja & Nepomuceno 2006). Additionally, rapid expansion, urbanization, and land-use conversion have led to the establishment of settlements closer to the lake shore, which may pose threats to the further degradation of the water quality (WAVES 2016).

The primary agency tasked with monitoring and managing the lake activities is the Laguna Lake Development Authority (LLDA). Quarterly water quality reports published by the agency consistently show high total and fecal coliform counts in most of its tributaries and some lake stations (LLDA 2018). However, current monitoring efforts do not generate any information regarding the origin of fecal contamination. Tracing the sources of pollution is useful in the development of management schemes for lake sustainability, evaluation of public health risks, and civil liability procedures (Hagedorn *et al.* 2011).

Microbial source tracking (MST) is a set of tools that uses various indicator organisms for the identification of dominant sources of fecal contamination in a system. Library-independent MST (LIM) utilizes organisms that inherently indicate their host sources or have an established niche (Hagedorn *et al.* 2011). *Cryptosporidium* is a protozoan parasite which has been implicated in numerous waterborne outbreaks of diarrheal disease. The presence of the parasite in surface waters poses a threat since they are highly resistant to common disinfection methods, require a low infectious dose, and are capable of zoonotic infection (Xiao *et al.* 2001).

There are over 40 described *Cryptosporidium* species and over 60 recorded genotypes which differ significantly in their sequences (Fayer 2010; Ryan *et al.* 2014). Research on *Cryptosporidium* molecular epidemiology suggests strong host specificity of its different species and genotypes (Cacciò *et al.* 2005; Hunter & Thompson 2005). Thus, it could be a model organism for LIM (Ruecker *et al.* 2007; Prystajecky *et al.* 2014).

In this study, we performed detection and genotyping of *Cryptosporidium* in Laguna Lake and river water samples collected over a 1-year period. Information on the occurrence and diversity of *Cryptosporidium* species sheds light into the extent of fecal contamination in the lake and the possible host sources. This study was conducted with the objective of providing means of monitoring *Cryptosporidium* contamination and distribution in the lake system for MST. To the best of our knowledge, this is the first study involving *Cryptosporidium* as an MST tool in the Philippines.

MATERIALS AND METHODS

Study site and sample collection

Surface water was collected within the Laguna Lake basin, in the provinces of Rizal, Laguna, and Metro Manila. The study focused on selected Laguna Lake stations and river tributaries known to have the highest total coliform counts based on the quarterly water quality monitoring reports prepared by the LLDA (2018) (Figure 1). Three Laguna Lake sampling stations (i.e. LS2, LS5, and LS8) represented the East, Northwest, and South Bay, respectively. The eight tributary monitoring stations selected (i.e. TR1 to TR8) were Bagumbayan River, Mangangate River, Sapang Baho River, Tunasan River located in the Metro Manila area; and Biñan River, Pila River, San Cristobal River, and Santa Rosa River located in Laguna, respectively. Based on observations during field collection, tributaries in the metropolitan area (i.e. TR1, TR2, TR3, and TR4) were noted to be surrounded by commercial and industrial activities, while southern tributaries (i.e. TR5, TR6, TR7, and TR8) are more dominated by agricultural, industrial, and livestock operations (Tanganco *et al.* 2019).

Sample collection in lake station samples was carried out on a monthly basis from March 2018 to April 2019.

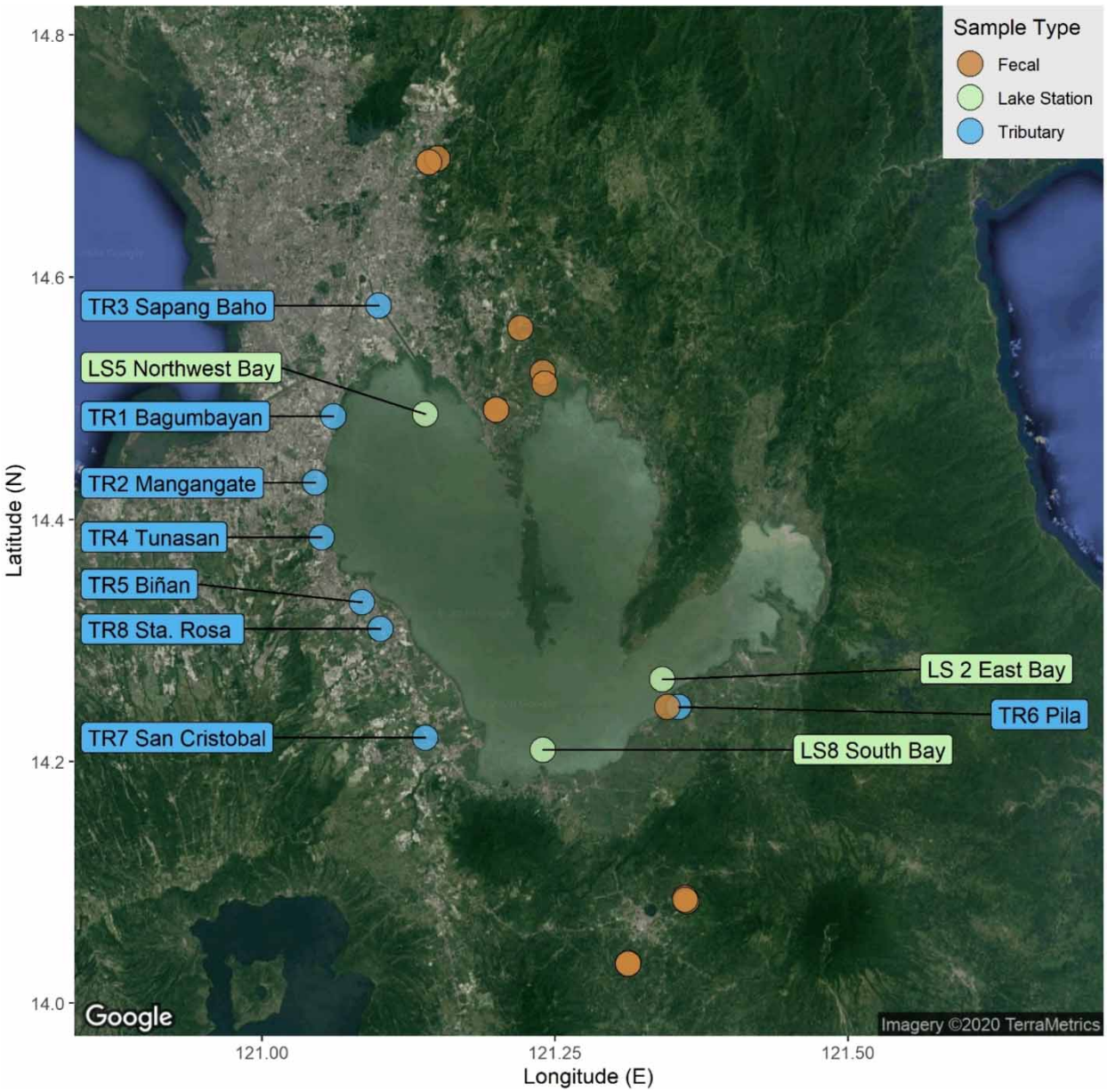

Figure 1 | Map of Laguna Lake and surrounding regions. Points indicate sites of tributary rivers, lake stations, and farms sampled in this study.

Sampling of tributary samples was conducted on at least four occasions per wet (May to October) and dry (November to April) seasons. A total of 68 tributary samples and 36 lake station samples were obtained. One-L surface water samples were collected from each sampling point and immediately transported under cold storage for further processing in the laboratory. Two replicates were obtained for each sampling point.

Besides the water samples, fecal samples were also collected from livestock and poultry farms near the study site, in the provinces of Laguna and Rizal. The animal hosts chosen were chickens, ducks, cows, and pigs, representing the common livestock raised in the area. Fecal sample collection from each host was conducted on at least three occasions per season. Approximately 10 g of feces were collected from individual animal coops in each farm. The

samples were pooled per host, and 3 g were collected for sample processing.

Sample processing and protozoa concentration

One-L surface water samples were processed by filtration using 47-mm mixed cellulose ester filters with 3.0 μm pore size (FilterBio, China) using a vacuum pump (Millipore, USA). The filters were then eluted using 0.01% Tween-80 and centrifuged for 10 min at 1,500 × *g* in order to pellet out the protozoan oocysts (Guy *et al.* 2003). Some tributary river samples exhibited high turbidity, for which pre-filtration was first conducted using a double layer of gauze. These samples were then processed following Robertson's protocol for collecting *Cryptosporidium* in wastewater samples by centrifugal concentration (Robertson *et al.* 2000). Briefly, 50 mL of 20% Tween 80 was added to a 1-L sample volume and continually mixed for 15 min. The samples were then centrifuged for 15 min at 2,000 × *g* and the supernatant carefully discarded. The resulting pellet was resuspended in 1 mL 1X phosphate-buffered saline (PBS) and was used for downstream analysis.

For the fecal samples, 3 g of feces from pooled samples of each animal type were weighed in a 15-mL conical tube and 10 mL PBS was added. The suspension was vortexed and sieved through a double layer of gauze. Afterward, 5 mL of 1 M sucrose solution was added before centrifugation at 2,000 rpm for 5 min. The resulting interphase (~3 mL) was aspirated and centrifuged again using the same conditions. The pellet was then resuspended in 1 mL 1X PBS and stored until further use.

Freeze-thaw and DNA extraction

Before DNA extraction, 15 cycles of freeze-thaw were performed on the sample pellets in order to break the oocyst walls (Nichols & Smith 2004). This was performed by submerging the tubes in an ethanol bath with dry ice (−78 °C) for 5 min, and immediately thawing using a heat block set at 95 °C. Extraction of nucleic acid was done using Invitrogen PureLink™ commercial kit following the manufacturer's instructions.

Cryptosporidium 18S rRNA gene PCR assays

Molecular detection of *Cryptosporidium* was conducted using a nested PCR assay targeting a hypervariable region of the 18S rRNA gene. For the first round, the primers NDIAGF2 and NDIAGR2 were used as described by Nichols *et al.* (2010), producing an expected length of 655–667 bp depending on the species of *Cryptosporidium*. Briefly, the PCR mix consisted of 1X GoTaq Green Mastermix, 200 nM of both forward and reverse primers, bovine serum albumin (BSA) at 400 μg/mL, nuclease-free water, and 1 μL of DNA in a 25 μL reaction volume. The primary PCR conditions consisted of the following step cycle protocols: initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 30 s, and final extension at 72 °C for 10 min. The secondary nested PCR used the *Cryptosporidium* diagnostic primers (CPB-DIAG) as an inner reaction producing 450-bp amplicons (Nichols *et al.* 2003). The PCR mix consisted of the same components, but 1.5 μL of the primary PCR product was used as DNA template. The secondary PCR followed the same protocol as the first round, with the exception of the annealing temperature lowered to 61 °C. The PCR assays were performed in triplicate along with positive and negative controls in each run.

Analysis of PCR product by gel electrophoresis

PCR products were run through a 2% agarose gel at 280 V for 30 min. The gels were then viewed at 260 nm (Bio-print ST4, Vilber Lourmat, UK), and bands were normalized using a 1 kb ladder (Hyperladder, Bioline, USA) as an external reference. Amplicons from the second round of nested PCR whose band size corresponded to the predicted size of 450 bp were deemed to be presumptively positive for *Cryptosporidium*. For confirmation, PCR products corresponding to the desired target size were sent to Macrogen Inc., Korea, for DNA purification and paired-end sequencing.

Determination of species and genotypes by sequencing

Obtained sequences were first trimmed and assembled into contigs by *de novo* assembly using Geneious Prime (Kearse

et al. 2012). The consensus sequences were then run through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for alignment of species and genotypes to reference sequences in the GenBank database. Sequences generated in this study were submitted to GenBank with accession numbers MT649670 to MT649683.

Phylogenetic analysis

Reference sequences of known *Cryptosporidium* species and genotypes were obtained from GenBank. Multiple alignment was performed via the Multiple Alignment using Fast Fourier Transform (MAFFT) v.7.450 (Katoh & Standley 2013) as implemented in Geneious Prime v.2019.0.4 (Kearse *et al.* 2012). Model testing was conducted in MEGA X as well as neighbor-joining tree construction using the Tamura 3-parameter (T92) model with gamma distribution and 1,000 bootstrap replicates (Tamura 1992). The output was rooted to the outgroup, *Eimeria tenella*.

Data analysis

All statistical analyses were performed using R version 3.6.3. A comparison of the occurrence of *Cryptosporidium* between wet and dry months was conducted using Fischer's exact test as implemented in R.

RESULTS

Frequency of detection of *Cryptosporidium* in Laguna Lake and its tributaries

A total of 104 surface water samples and 50 fecal samples were collected over a 1-year monitoring period to investigate the presence of *Cryptosporidium* spp. Eleven water samples (Table 1) and two fecal samples (Table 2) were positive for *Cryptosporidium* as confirmed by PCR and sequencing.

Tributary samples had a higher frequency of detection (14.71 ± 8.49%, 95% CI) compared with lake stations (2.78 ± 5.46%, 95% CI). A total of 8 water samples were positive during the wet season (14.29 ± 9.25%, 95% CI) and three during the dry season (6.25 ± 6.93%, 95% CI); a difference which was not statistically significant (Fisher's exact test, $P = 0.217$). Only one lake station site was positive for *Cryptosporidium* (South Bay – 8.3%). Pila River had the highest rate of occurrence with three positive samples (33%), followed by Biñan River (28.6%), and Santa Rosa and Bagumbayan Rivers (22.2%), with two occurrences each. No parasites were detected in Mangangate, Tunasan, and San Cristobal rivers.

Identification of *Cryptosporidium* genotypes

Cryptosporidium parvum was the most frequently detected species in all the samples, appearing in tributary samples (Pila River and Sta. Rosa River), a lake station sample (South Bay), and in one cattle fecal sample. For one lake station sample (LS8), multiple runs of sequencing from replicate PCR assay results revealed two distinct sequences, although both were identified as *C. parvum*. *C. muris* was also detected twice in tributary rivers (Bagumbayan River and Biñan River). Other species and genotypes were detected once (*C. hominis, C. galli, C. suis, C. baileyi,* and *Cryptosporidium* rat genotype IV) in the tributaries. *C. baileyi* was also detected in a duck fecal sample. Nucleotide sequences of the PCR products show high sequence similarity (97–100%) with reference sequences. The constructed phylogenetic tree using the 18S rRNA gene supports the identification of the species as they formed well-defined clusters with their determined species and genotypes (Figure 2), despite some sequences (*C. parvum* LS8, *C. hominis* TR1, *Cryptosporidium* rat genotype IV TR8, and *C. baileyi* TR6) having distinct point mutations compared with reference sequences.

DISCUSSION

The parasite *Cryptosporidium* is one of the leading causes of diarrhea worldwide. About 40 species and genotypes of *Cryptosporidium* are recognized, which have distinct host ranges and virulence (Feng *et al.* 2018). Currently, knowledge of *Cryptosporidium* species and genotypes in the Philippines is limited (Labana 2019). Most studies reporting the occurrence of *Cryptosporidium* in the country do not provide sequencing data for molecular identification.

Table 1 | Frequencies of *Cryptosporidium* detection in Laguna Lake and tributary river samples and their species/genotypes

	Wet season (May to October) $n = 56$		Dry season (November to April) $n = 48$		
Sample source	**Occurrence (No. of positive/total samples)**	**Species/genotypes detected**	**Occurrence (No. of positive/total samples)**	**Species/genotypes detected**	**Total 10.57%**
Lake Station	5.56% (1/18)		0% (0/18)		2.78% (1/36)
LS2	(0/6)	ND	(0/6)	ND	ND
LS5	(0/6)	ND	(0/6)	ND	ND
LS8	(1/6)	*C. parvum*	(0/6)	ND	8.3% (1/12)
Tributaries	18.42% (7/38)		10.00% (3/30)		14.71% (10/68)
TR1	(1/5)	*C. muris*	(1/4)	*C. hominis*	22.2% (2/9)
TR2	(0/4)	ND	(0/4)	ND	ND
TR3	(1/5)	*C. suis*	(0/4)	ND	11.1% (1/9)
TR4	(0/5)	ND	(0/4)	ND	ND
TR5	(2/5)	*C. galli, C muris*	(0/2)	ND	28.6% (2/7)
TR6	(2/5)	*C. parvum, C. baileyi*	(1/4)	*C. parvum*	33% (3/9)
TR7	(0/4)	ND	(0/4)	ND	ND
TR8	(1/5)	*C. parvum*	(1/4)	*Cryptosporidium* spp.	22% (2/9)

ND, not detected.

Table 2 | Frequencies of *Cryptosporidium* detection in fecal samples and their species/genotypes

Location of sampling point	Animal host	Number of positive samples/total number of samples ($n = 50$)	Species/genotype detected
Laguna	Chicken	0/7	ND
	Cow	0/7	ND
	Duck	1/7	*C. baileyi*
	Pig	0/7	ND
Rizal	Chicken	0/6	ND
	Cow	1/5	*C. parvum*
	Duck	0/5	ND
	Pig	0/6	ND

ND, not detected.

Here, we provide a method for the molecular surveillance of *Cryptosporidium* that can be adapted to the current water quality management efforts in Laguna Lake, for the purposes of (1) identifying fecal contamination sources and (2) monitoring the presence of the pathogen, which can have public health implications. In this study, we report the application of *Cryptosporidium* as a model organism for library-independent MST. By sequencing the 18S rRNA gene, we were able to determine species or genotypes present in aquatic environmental samples. Knowledge of the host specificity of certain species would reflect the possible sources of contamination dominant in the water system. This study also provides information on the occurrence and distribution of *Cryptosporidium* in Laguna Lake and its tributaries.

The presence of *Cryptosporidium* contamination in the vicinity of Laguna Lake has already been established by previous studies. Rivera & Yason (2008) reported the occurrence of *Cryptosporidium* in farm animals from Laguna and Batangas, with pigs and calves having the highest rate of infection at 34.3 and 20.4%, respectively. Wild rats and mice from Los Baños (31.8%) and Calauan (63%), Laguna, also had high prevalence of *Cryptosporidium* (Ng-Hublin *et al.* 2013). In Asian clams propagated in Laguna Lake, 20% were positive for *Cryptosporidium* (Paller *et al.* 2013). Paller *et al.* (2017) also reported 100% prevalence of *Cryptosporidium* in recreational waters in Calamba, Laguna. A recent report by Masangkay *et al.* (2020a) confirms the presence of *Cryptosporidium* in the surface and bottom waters of Laguna Lake, as well as in other freshwater reservoirs in Luzon Island.

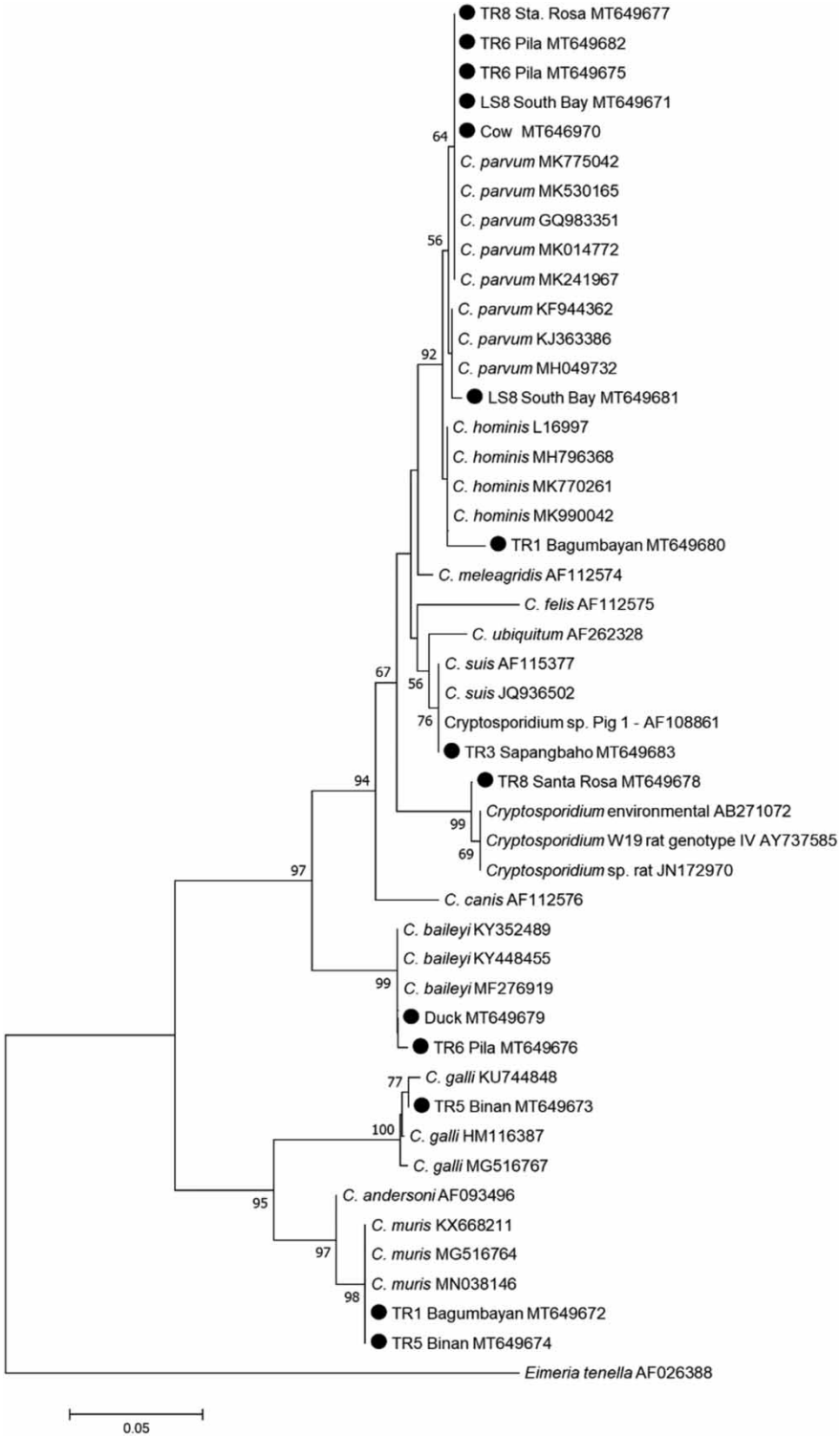

Figure 2 | Phylogenetic relationship of partial small subunit rDNA sequences from *Cryptosporidium* isolates generated from this study and known species/genotypes retrieved from GenBank. Evolutionary distances between sequences were inferred using neighbor-joining analysis calculated by the Tamura 3-parameter model with gamma distribution. The consensus tree inferred from 1,000 bootstrap replicates shows bootstrapping values (>50). Reference sequences are shown with accession numbers.

The overall rate of occurrence in water samples was 10.57%, being detected more frequently in tributary rivers. Other studies of *Cryptosporidium* occurrence in environmental water sources in the Philippines reported 50% in river samples from Manila, Pampanga, and Cavite, 33.3% in a lake in Batangas (Onichandran *et al.* 2014), 77.3% in raw water samples from the La Mesa Dam (Masangkay *et al.* 2016), and 22.2% in a river in Ifugao (Labana *et al.* 2018). The lower incidence of *Cryptosporidium* in the lake samples may be due to the dilution of fecal pollution. Detection of *Cryptosporidium* DNA was unsuccessful in some tributaries as well (Mangangate, Tunasan, and San Cristobal rivers), which possibly indicates a lower concentration of oocysts. We recommend that larger volumes of water be obtained in further studies in order to have a higher likelihood of capturing oocysts.

Detection of *Cryptosporidium* was more frequent during the wet season (14.3%) than dry season (6.25%). Multiple species identification also occurred only during the wet season, indicating different inputs of fecal pollution during this period. This agrees with previous findings of higher prevalence of oocysts during increased rainfall events due to increased oocyst transport mechanisms and resuspension (Atherholt *et al.* 1998; Jiang *et al.* 2005). A study by Paller *et al.* (2013) cited rainfall and water clarity to be significantly associated with the occurrence of *Cryptosporidium* from Asian clams in Laguna Lake, two factors that are also implicated with the increase of *Cryptosporidium* waterborne outbreaks (Rose *et al.* 2002; Lal *et al.* 2013). The incidence of cryptosporidiosis in Philippine patients was also reported to have an increasing trend during the rainy season (Natividad *et al.* 2008).

C. parvum was the most frequently detected genotype in positive samples (45%), suggesting that it may be the dominant species in the tributary and lake waters. The species was also detected in a cow fecal sample. The zoonotic species *C. parvum* is known to mainly infect humans and cows (Xiao 2010), which suggests both human and/or livestock contamination in agreement with the urban and rural landscape of the study site. The species *C. hominis*, which is known to be exclusively infective to humans, was detected in Bagumbayan River. *C. parvum* and *C. hominis* represent the two species responsible for most human cases of *Cryptosporidium* (Xiao 2010). The presence and dominance of human-infective species in the water samples suggest that human fecal contamination, or untreated sewage, may be significant contributors of pollution in the lake system. This has implications for public health and water security, particularly since Laguna Lake is also used as a raw water resource. The two human-infective species have also been reported by Masangkay *et al.* (2020b) in lakes across three major islands of the Philippines, further highlighting the need to strengthen water quality management efforts.

Two samples were identified as *C. muris* and one sample as rat genotype IV (Jiang *et al.* 2005), which have rodents as their major hosts. This suggests the presence of fecal contamination coming from non-point sources, including wildlife or sewage discharge since *C. muris* can be prevalent in wastewater (Zhou *et al.* 2003). Aside from wild rats (Zhao *et al.* 2018), the rat genotype IV (previously W19) has been detected in several environmental samples such as raw water (Feng *et al.* 2009; Chalmers *et al.* 2010) and a watershed in Canada (Ruecker *et al.* 2013). However, further characterization of this genotype is necessary to establish host range and distribution. A previous study of *Cryptosporidium* genotypes from rodents in neighboring Los Baños and Calauan, Laguna, reported the presence of *C. muris* and *Cryptosporidium* rat genotype IV (Ng-Hublin *et al.* 2013). Other identified species point to agricultural contamination from poultry (*C. galli* and *C. baileyi*) and swine (*C. suis*). These results suggest that farm animals and wildlife may be important reservoirs of *Cryptosporidium* in the Laguna Lake system. It is noteworthy that although these animal-derived species are commonly thought to have a narrow host range, some have been reported in humans (Ryan *et al.* 2014; Zahedi & Ryan 2020), in particular, *C. muris* (Feng *et al.* 2011) and *C. suis* (Xiao *et al.* 2002; Leoni *et al.* 2006; Cama *et al.* 2007; Wang *et al.* 2013). These zoonotic species present a valid public health concern as they are able to infect both immunocompetent and immunocompromised persons (Ryan & Hijjawi 2015).

Knowledge of the sources of *Cryptosporidium* in the watershed aids in focusing the efforts of mitigating fecal contamination in the area. The diverse host sources found in the sampling points and differing land use indicate that management requires different strategies. The land use in the southeastern portion of the watershed is mainly agricultural,

compared with the western side which features highly urbanized and built-up land cover (Tanganco *et al.* 2019). For example, the highest occurrence of *Cryptosporidium* was reported in Pila River (33%) and the identified species (*C. parvum* and *C. baileyi*) point to agricultural sources. Interestingly, the same species identified in Pila (*C. baileyi)* was also detected in a duck fecal sample that was obtained in a nearby sampling point, further supporting the evidence of contamination from poultry sources. The high occurrence of *Cryptosporidium* in Pila is likely due to the expansive farming areas in the location. In contrast, human-infective (*C. hominis* and *C. parvum*) and sewage-associated species (*C. muris,* rat genotype IV) were found on the western sampling points (Bagumbayan, Biñan, Sta. Rosa). A deeper understanding of fecal pollution sources, transport, and distribution is required in order to properly assess environmental and public health implications threatening the water supply security. We recommend the adoption of MST with current water quality monitoring technologies to augment pollution mitigation measures.

CONCLUSION

This study reports the occurrence of *Cryptosporidium* species in surface waters of Laguna Lake. The present work demonstrates the application of *Cryptosporidium* monitoring and genotyping as a method for MST. Different host-adapted species and genotypes of *Cryptosporidium* were identified, showing the dominant sources of fecal contamination in Laguna Lake and its tributaries. The findings also underscore possible health risks caused by infection with pathogenic and zoonotic species of *Cryptosporidium*. This empowers local government and management agencies to enact measures for the mitigation of fecal pollution to protect human and animal health, such as the inclusion of protozoan limits in the water quality standards.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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