Research Article

Increased Detection of *Cryptosporidium* and *Cyclospora* spp. Oocysts in a Major Philippine Watershed Following Rainfall Events

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ABSTRACT

Waterborne Coccidians are emerging pathogens and are not entirely known in developing countries. *Cryptosporidium* and *Cyclospora* have been reported to cause diffuse watery diarrhea among immunocompetent and immunocompromised individuals alike but are not included in routine water quality examinations and medical diagnosis thereby leading to being underreported, undiagnosed or misdiagnosed and neglected as agents of intestinal-protozoan ailments. Water samples from a major watershed in Metro Manila, Philippines were investigated. A total of 99 samples (50-mL each), were filtered to obtain sediments that were processed and smeared onto glass slides and stained using modified Kinyoun's acid-fast technique and microscopically observed for *Cryptosporidium* and *Cyclospora* oocysts. The 3-day collection (33 samples per day) returned positive results: Day 1 returned 42% (14/33) positive water samples, Day 2 with 85% (28/33) and Day 3 with 88% (29/33) for *Cryptosporidium* and *Cyclospora* oocysts. Overall, oocyst positivity was 72% (71/99). The results of this study support previous Coccidian findings in a major Philippine watershed and calls for further exploration of source water samples for intestinal protozoan pathogens, the identification of which can lead to initiatives in improving water quality assessment and the prevention of the transmission of waterborne protozoan pathogens to the general public.

Key words: Coccidian, *Cyclospora, Cryptosporidium*, Watershed, Philippines.

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INTRODUCTION

Most parasitic infections are found in developing tropical or subtropical countries and cause a tremendous burden of disease. Some of these parasites that not restricted by extreme environments and remain viable even outside of the host are intestinal Coccidians like *Cryptosporidium* spp. and *Cyclospora* spp., which are classified as waterborne protozoans that cause significant burden of intestinal ailment.[1,2]

Cryptosporidium spp. is a major public health concern in both developing and industrialized countries and is a major cause of morbidity and mortality in immunocompromised patients.[3] *Cryptosporidium* is easily transmitted through water due to its high resistance to chemicals and can survive in harsh environments from weeks to months.[4] *Cryptosporidium* oocysts are particularly more resistant than other protozoan cysts in removal and inactivation by conventional water treatment such as coagulation, sedimentation, filtration and chlorine disinfection.[5] Meanwhile, Cyclosporiasis has also been reported among immunocompromised hosts, primarily from AIDS patients, in which it causes severe manifestations. A year-long study conducted in the Philippines was able to isolate one stool sample positive for *Cyclospora cayetanensis* and *Isospora belli*. This rare occurrence can be easily overlooked and thus highlighted the awareness on detecting the said parasites.[6]

The first report of *Cryptosporidium* and *Cyclospora* from source water in the Philippines was submitted in 2016 and has since aroused interest in the identification of

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waterborne protozoan pathogens in the country.[7] The aims of this study were to further establish the presence of waterborne Coccidians in Philippine watersheds and provide the necessary data for the formulation of government initiatives in improving water quality assessment and the safeguarding of the public against the transmission of waterborne protozoan pathogens.

MATERIALS AND METHODS

Study site and water sampling

The Angat Watershed Reservation (14°54'39"N 121°09'37.8"E) is a protected reservation and the only major watershed in the country that has a remaining rainforest reserve of 62,309 hectares in Metro Manila and provides 97% of the Metro Manila water supply. Its water output is directly funnelled to Ipo Dam and then passed through La Mesa Dam Aqueducts for treatment and then into domestic and industrial distribution.[8] Figure 1 shows the Raw Water Conveyance Map of the Metropolitan Waterworks and Sewage System (MWSS) in Luzon, Philippines and at the heart of the watershed system in the National Capital Region is Angat Dam (Figure 2b) where the water samples were collected. A total of 99 surface water samples (50-mL each) were obtained for a period of three days (33 samples per day from July 28 to 30, 2016.) from three intakes (11 per intake) using a sterile polyethylene cup attached to a rod in the following coordinates: Area 1-14°91' 16.8"N, 121°16'23.9"E, Area 2-14°91' 31.8"N, 121°17'07.4"E and Area 3-14°91' 50.6"N, 121°17'96.2"E. The collected water samples were placed in an ice chest to be transported to the laboratory for processing within 24 hr.

Processing, Microscopy and Validation

Surface water samples were manually processed following the protocols of Masangkay *et al*. 2016.[7] Briefly, the water samples were manually filtered using glass-microfiber filter (1.2-µm pore size) fitted inside 50-mL disposable syringe. Sediments were collected and eluted to a volume of 5-mL using sterile distilled water and concentrated by centrifugation at 1500 g for 15 min.[9] Pellets were recovered and prepared into 2-mL suspensions and stored in microtubes prior to smearing. 25-µL pellet suspensions were prepared into 1 X 1 cm diameter smears on glass slides and stained with Modified Kinyoun's stain. 200 oil immersion fields were examined for *Cryptosporidium* and *Cyclospora* oocysts.[10] Suspected *Cryptosporidium* and *Cyclospora* oocysts were compared to published images found in CDC DPDx.[11,12] Positive slides were sent to the Parasitology Department, Research Institute of Tropical Medicine-Department of Health for the second round of microscopic examination and external validation.

RESULTS

As shown in Tables 1 and 2, there was an increasing trend of positivity for both *Cryptosporidium* and *Cyclospora* oocysts during the three-day sampling period where *Cryptosporidium* was more frequently detected at 58% (57/99) compared to *Cyclospora* at 45% (45/99) as can be referenced in Table 3. As shown in Table 2, with respect to location, Area 3 had the highest number of positive samples at 55% (6/11) on the first day of collection.

Table 1: Summary of daily reports of positivity for *Cryptosporidium* and *Cyclospora* spp. oocysts.

Day	Samples	Positive Samples	*Cryptosporidium* spp. oocysts		*Cyclospora* spp. oocysts	
Day 1	33	42%	12	36%	7	21%
Day 2	33	85%	21	64%	18	55%
Day 3	33	88%	24	73%	21	64%

Note: Positive samples are either positive for *Cryptosporidium* spp. or *Cyclospora* spp. oocysts or both.

Table 2: Summary of water samples positive for target Coccidians per sampling area.

	Day 1		Day 2		Day 3	
	Positive	(%)	Positive	(%)	Positive	(%)
Area 1	4/11	36%	11/11	100%	11/11	100%
Area 2	4/11	36%	8/11	73%	10/11	91%
Area 3	6/11	55%	9/11	82%	8/11	73%

Note: Positive water samples are either positive for *Cryptosporidium* spp. or *Cyclospora* spp. oocysts or both.

Table 3: Frequency of *Cryptosporidium* spp. and *Cyclospora* spp. oocyst from water samples.

	***Cryptosporidium* spp. oocysts**		***Cyclospora* spp. oocysts**	
	Frequency	(%)	Frequency	(%)
Total positive	57	58%	46	46%

Table 4: Summary of frequency of detection of target Coccidians from Angat Dam over a 3-day period.

Day	Daily Samples	Positive samples	(%)
Day 1	33	14	42%
Day 2	33	28	85%
Day 3	33	29	88%
Total positive samples		71	71%

Note: Positive samples are either positive for *Cryptosporidium* spp. or *Cyclospora* spp. oocysts or both.

Table 5: Breakdown of the daily report for *Cryptosporidium* and *Cyclospora* spp. oocysts per sampling area.

	Samples	***Cryptosporidium* spp.**		***Cyclospora* spp.**	
		Frequency	(%)	Frequency	(%)
Day 1					
Area 1	11	3	27%	3	27%
Area 2	11	3	27%	3	27%
Area 3	11	6	54%	1	9%
Day 2					
Area 1	11	7	63%	11	100%
Area 2	11	5	45%	7	63%
Area 3	11	9	81%	0	0%
Day 3					
Area 1	11	11	100%	7	63%
Area 2	11	8	72%	8	72%
Area 3	11	5	45%	6	54%

Area 3 is the farthest sampling area from the catch-basin (Area 1) and suggests that high density of oocysts was accumulated in this location compared to other sampling areas prior to the sample collection. However, Area 1 presented with the highest number of positive samples at 100% for both day 2 and day 3 collection. This can be attributed to precipitation or rainfall events during day 2 and day 3 collection that may have facilitated the flow and redistribution of oocysts to the catch-basin area. Table 4 submits the positivity of Angat Dam for target Coccidians in the study where 71% (71/99) positivity was recorded over a three-day sampling period for either *Cryptosporidium* spp. or *Cyclospora* spp. oocysts or both where on the average, day 3 has the highest rate of positivity at 88% (29/33) which again suggests that the rainfall event redistributed the oocysts from terrestrial areas to the surface waters of Angat Dam through run-off of contaminated soil and the waters subsequent flow to sampling area 1. Table 5 shows the consistent detection of target Coccidians from the surface waters of Angat Dam over a three-day sampling period which suggests high contamination of the water reservoir with potential waterborne protozoan pathogens.

All stained slide preparations were preliminarily read by licensed medical technologists with comprehensive training in identification of parasites of public health importance and were sent to the Parasitology Department of the Research Institute for Tropical Medicine-Department of Health for final verification and confirmation (external validation). Figure 3 presents a graphical representation of the increased isolation of *Cryptosporidium* and *Cyclospora* oocysts possibly influenced by precipitation events following day 1 of sampling. Figure 4. Shows *Cryptosporidium* spp. oocyst detected from the samples AD-2-1, AD-2-21, AD-3-7 and *Cyclospora* spp. oocysts detected from samples AD-3-1, AD-1-11 and AD-3-5 as observed under 1000X magnification using oil immersion objective (OIO). Staining characteristics, oocyst morphology and diameter were used to identify *Cryptosporidium* spp. and *Cyclospora* spp. oocysts for comparison with published images from CDC DPDx. *Cryptosporidium* spp. oocysts are smaller than *Cyclospora* spp. oocyst with average measurements of 4 to 6 and 8 to 10-µm, respectively.

DISCUSSION

The Presence of *Cryptosporidium* oocysts in dams, watersheds, or water reservoirs, have been well-documented in different parts of the globe.[13-15] Although *Cyclospora* has been mainly isolated in fresh produce,[16-18] reports have also been submitted for its isolation from source water samples as well.[19,20] The presence of *Cryptosporidium* and *Cyclospora* spp. oocysts in the present study support the findings of the 2016 first report of the presence of *Cryptosporidium* and *Cyclospora* in a major Philippine watershed[7] where a three-day sampling period yielded 71% and 37% positivity for *Cryptosporidium* and *Cyclospora* spp. oocysts, respectively which are relatively close to 58% and 46% (Table 3) positivity for *Cryptosporidium* and *Cyclospora*, respectively, in the present study. As can be referenced in Figure 1, the accumulation of higher concentrations of the target Coccidians in La Mesa dam in the 2016 study by Masangkay *et al.* may have been contributed by the source water coming from Angat Dam.

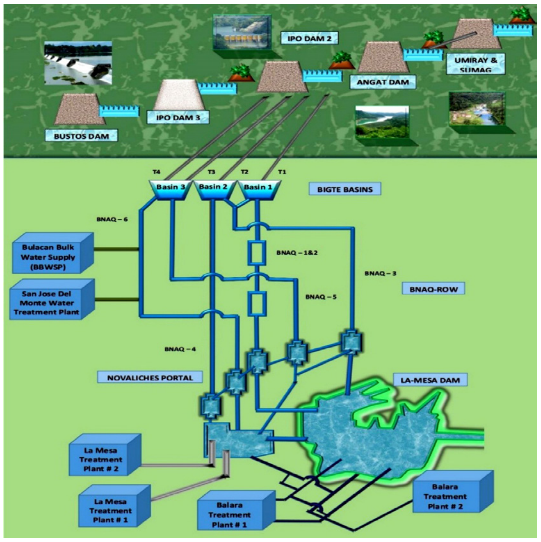

Figure 1: Metropolitan Waterworks and Sewage System (MWSS) Raw Water Conveyance Map (Source: http://mwss.gov.ph/learn/metro-manila-water-supply-system/).

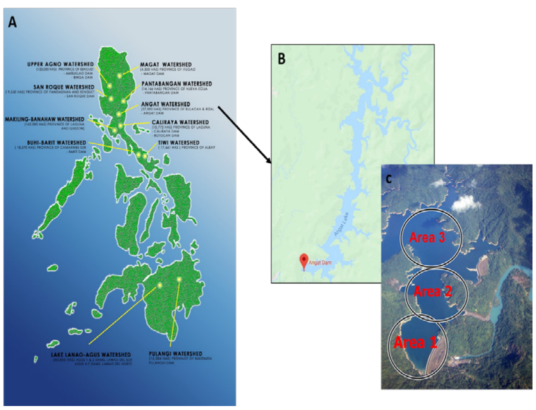

Figure 2: Locations of major watersheds in the Philippines and sampling areas of the study. (a) Major watersheds in the Philippines (Source: https://www.napocor.gov.ph/npcwatershed/index.php/about-us/north-luzon-watershed/angat-watershed); (b) Angat dam (Source: Google maps); (c) Angat dam sampling areas.

The general profile of source water environments are grazing and forest grounds inhabited by a plethora of animal species, several of which have been reported for the presence of *Cryptosporidium* like ungulates,[21] reptiles,[22,23] birds, amphibians and fishes.[24] Point sources of *Cyclospora cayetanensis*, which is the *Cyclospora* species of human importance has only been isolated from humans[25] thereby tracing its spread in water sources from contamination of the same with human waste. Precipitation also plays a role in the contamination of source waters with potentially pathogenic Coccidian species thru soil-run off, where feces contaminated soil is deposited in environmental water sources following rainfall events.[26,27] This was consistently observed in both the 2016 study by Masangkay *et al.* and this present study by the increased detection of both *Cryptosporidium*

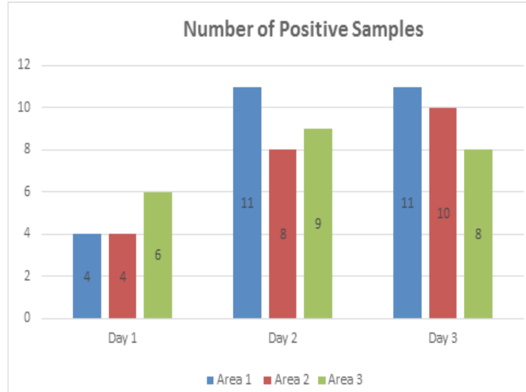

Figure 3: Increased isolation of target organisms as the days of collection proceeded with rainfall events taking place in the research locale and study site (Day 2 and Day 3).

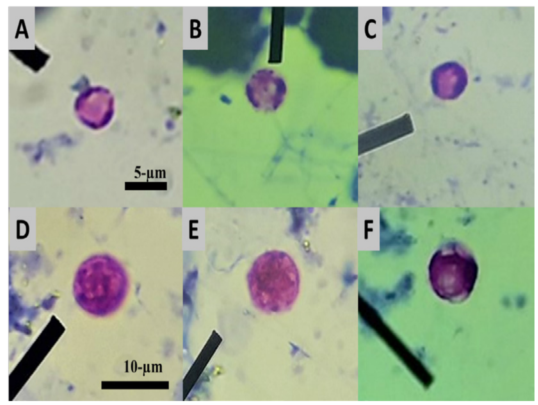

Figure 4: A to C: *Cryptosporidium* spp. oocysts; D to F: *Cyclospora* spp. oocysts; A to F: 1000X magnification; A AD-2-1; B AD-2-21; C AD-3-7; D AD-3-1; E AD-1-11; F AD-3-5. *Cryptosporidium* and *Cyclospora* spp. oocyst findings were externally validated by The Parasitology Department, Research Institute for Tropical Medicine-Department of Health.

and *Cyclospora* spp. oocysts on Day 2 and 3 of the sampling period.

The oocysts present in environmental waters can be accumulated by biological indicators which can for a time trap waterborne protozoan pathogens and maintain its viability, making it available for further spread and transmission to humans. This was exemplified in the cases of *Cryptosporidium* accumulation in marine bivalves in the Philippines[28] and the first report of *Cryptosporidium hominis* in substrate-associated biofilms.[29]

Although recent studies report that immunocompetent individuals are relatively protected from the adverse effects of *Cryptosporidium* and *Cyclospora*, significant intestinal ailments have been reported from immunocompromised individuals; making the infants, the young, the old, HIV and organ transplant population among others, susceptible to disease states.[30-32] Whichever the case may be, the detection of the presence of *Cryptosporidium* and *Cyclospora* spp. oocysts in Philippine source waters warrant support for further and in-depth explorations.

CONCLUSION

Potentially pathogenic Coccidian oocysts are present in Philippine watersheds. The contamination of food and drinking water, or the accidental ingestion of water during recreational activities that contain *Cryptosporidium* and *Cyclospora* can potentially lead to otherwise, avoidable gastrointestinal ailments or worse, mortality. Precipitation events seemingly increase the frequency of detection of waterborne protozoan pathogens in water samples, owing to contamination of environmental waters with soil run-off contaminated with human and animal feces. Support is necessary for further exploring the quality of Philippine freshwater sources relative to waterborne protozoan pathogens and the elaboration of the same concerning its transmission and pathogenicity.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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