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Water-borne coccidians in Philippine water sheds : A national inceptive study

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Abstract

Water-borne coccidians, one of few emerging pathogens, are not entirely known in developing countries. It has caused illnesses mainly on immunocompromised hosts but can also cause pathology in immunocompetent individuals. Due to the rarity of well-documented occurrences of these pathogens, water samples from a major watershed in Metro Manila, Philippines were investigated. 75 samples, 60 mL each, were filtered to obtain sediments. The sediments were processed and smeared onto glass slides and stained using modified Kinyoun acid-fast technique and microscopically observed for Cryptosporidium and Cyclospora oocysts. The 3-day collection (25 samples per day) yielded positive results: Day 1 produced 19 positive water samples (76%); Day 2 with 17 (68%); and Day 3 with 22 (88%) for Cryptosporidium and Cyclospora oocysts. Overall, oocyst positivity was 58 (77%). Measured physicochemical properties included pH, turbidity, total dissolved solids, fluoride, chloride, nitrate, and conductivity. This study was able to establish an inceptive profile of the presence of *Cryptosporidium* spp. and *Cyclospora* spp. oocyst in one of the Major water sheds in the National Capital Region of the Republic of the Philippines.

Key words : Coccidian, Cryptosporidium, Cyclospora, Watershed

INTRODUCTION

Parasitic infections, although distributed worldwide, thrive in countries with temperate climates. Infections can be acquired through a number of ways and preventive measures are often not sufficient enough to avoid them. Despite the fact that there are available treatments, eradication of parasites is more beneficial in the long-term. There are, however, some parasites that are not restricted by extreme environments and remain viable even outside of the host. Some of these parasites are intestinal Coccidians, *Cryptosporidium* spp. and *Cyclospora* spp., classified as water-borne protozoans that are emerging pathogens of global public health concern [1].

In 1976, the first cases of human infection by *Cryptosporidium* spp. were isolated from infrequent occurrences in immuno-compromised patients. While there is an estimated prevalence of infection with *Cryptosporidium parvum* of about 2% in immunocompetent patients with diarrhea in industrial countries, the average is about 22% in AIDS patients in developing countries[2].*Cryptosporidium* spp. have been found to infect a wide variety of animals with an epidemiology similar to that of giardiasis while *Cyclospora* spp. has been recovered from patients in several countries [3] both organisms having had identified histories of outbreaks in different parts of the globe. These outbreaks are attributed to the infective form of called oocyst. The oocysts are transmitted via ingestion of contaminated water or through fecal-oral route and are easily missed in diagnosis due to lack of special testing [4, 5] and is in fact not even included as part of routine testing for fecal analysis. The diagnosis of Cryptosporidiosis and Cyclosporiasis is based mainly on detection of the typical oocysts in stool specimens by use of an acid-fast stain[6]. Cryptosporidiosis causes diarrhea and are considered self-limiting, although there have been some cases of disseminated infection involving the lungs and gallbladder in persons with compromised immune responses. *Cyclospora* spp. causes infection similar to *Cryptosporidium* spp., except that it is common to relapse. *Cyclospora* spp. have been implicated in food-borne outbreaks [7, 8], making ingestion of food contaminated with oocysts, the primary mode of transmission. Although food-borne Cyclosporiasis outbreaks have not been linked to the consumption of any of the seafood items, most of the reports originate from coastal regions near both fresh and marine waters[9].Because of the low prevalence of Cryptosporidiosis and Cyclosporiasis in many areas, and the difficulty of testing for the presence of the parasites, examination for these particular parasites is often not routinely performed[10, 11]. On this note It is recommended that tests for Cryptosporidium be done as part of a general diarrhea screen during standard stool tests in diagnostic laboratories[12].

Several sources of drinking water in South-East Asia have been positive for numerous protozoan and amoebic species of public health concern [13]. Thus, it is imperative to establish a baseline Coccidian profile in major sources of drinking water namely: watersheds. The La Mesa Watershed Reservation (14°44′37″N 121°6′3″E) is the starting point of this National inceptive study; it is a protected reservation and the only major watershed and last remaining rainforest of its size 2,659 hectares (6,570 acres) in Metro Manila.

METHODS

Water Sampling

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 La Mesa Dam (Figure 2.) where the water samples were collected. La Mesa Dam along with Angat Dam and Ipo Dam, are the main sources of Metro Manila's water supply. They distribute water to thousands of households for domestic and industrial uses in Metro Manila. It is located in the

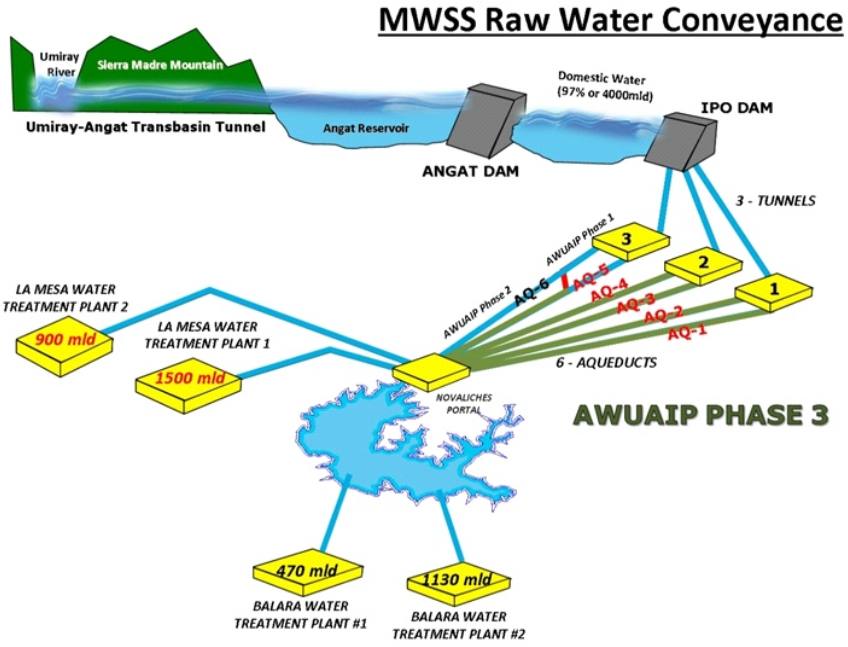

Fig. 1: PMetropolitan Waterworks and Sewage System (MWSS) Raw Water Conveyance Map http://mwss.gov.ph/awuaip3-2/

Fig. 2: La Mesa Watershed and Ecological Park
http://newsinfo.inquirer.net/223561/la-mesa-dam-on-high-alert-for-possible-overflow

La Mesa Watershed reservation in Quezon City. Surface water samples were obtained from the intakes by attaching sterile polyethylene cups to an expanding rod with a clamp at the end, and dipped 10-20 cm deep into the water until the cup is filled. The cups were then sealed, labelled and stored in an ice chest for transport.

Filtration

Water samples were filtered using Whatman Glass Microfibre filters (47 mm diameter, 1.2 µm pore size, Millipore). 50 ml syringe was used, with the Millipore placed at the base. The syringe was then filled with the water sample and the plunger was inserted and was gently pressed (to avoid causing damage to the filter) [14, 15].

Table 1. Daily results of water sampling					
Day	Samples	Positive for *Cryptosporidium* spp.		Positive for *Cyclospora* spp.	
		Frequency	(%)	Frequency	(%)
Day 1	25	17	68	10	40
Day 2	25	15	60	8	32
Day 3	25	21	84	10	40

Table 2. Total positivity of *Cryptosporidium* spp. and *Cyclospora* spp. oocyst from water samples				
	Cryptosporidium spp.		*Cyclospora* spp.	
	Frequency	(%)	Frequency	(%)
Total positive	53	71	28	37

Table 3. Positive water samples per intake						
	Intake 1		Intake 2		Intake 3	
	Positive	(%)	Positive	(%)	Positive	(%)
Day 1	8	100	5	56	6	75
Day 2	4	50	7	78	6	75
Day 3	6	75	9	100	7	88
Intake 1 = 8 samples; Intake 2 = 9 samples; Intake 3 = 8 samples						

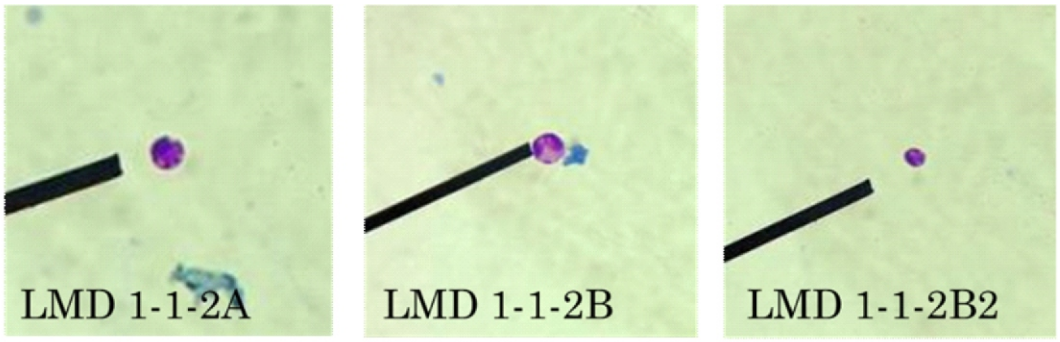

Fig. 3: *Cryptosporidium* spp. oocyst confirmed by the Research Institute for Tropical Medicine

Elution

After filtration of water samples, the Millipore filter was recovered and placed in a sterile 200 ml beaker. The Millipore filter was washed with 5 ml distilled water and the surface of the Millipore was gently scraped to release all the sediments to the eluent without damaging the oocyst.

Concentration

The eluent was then transferred to a 15 ml polyethylene conical tube and was centrifuged at 3,500 G for 15 minutes. After centrifugation, the supernatant was aspirated leaving the sediment to be stained and analyzed.

Staining and Microscopy

50 ul eluent was smeared and spread evenly at the center of each glass slides which was then heated in a dry oven at 50^0 Centigrade until completely dried. After dry-heating, methanol was used to fix the smears. Slides were dry-heated again for three minutes. Slides were stained using Modified Kinyoun Technique by flooding the smear area with Carbol Fuchsin for 10 minutes. Decolorization was done by flooding with 2% Sulfuric acid and 95% ethanol solution (1:1) and counterstained with methylene blue for one minute. 300 oil immersion fields (OIF) were examined for each slide using light microscopy.

RESULTS

Cryptosporidium spp. and *Cyclospora* spp. oocyst

Table 1. presents the Daily results of water sampling from the intakes. Findings for the first day showed that 19 out of the 25 samples were positive for either *Cryptosporidium* spp. oocysts or

Table 4. Positive samples for Day 1					
Intake	Samples	*Cryptosporidium* spp.		*Cyclospora* spp.	
		Frequency	(%)	Frequency	(%)
1	8	7	88	6	75
2	9	5	55	1	1
3	8	5	63	3	3

Table 5. Positive samples for Day 2					
Intake	Samples	*Cryptosporidium* spp.		*Cyclospora* spp.	
		Frequency	(%)	Frequency	(%)
1	8	3	38	2	25
2	9	6	67	3	33
3	8	6	75	3	38

Table 6. Positive samples for Day 3					
Intake	Samples	*Cryptosporidium* spp.		*Cyclospora* spp.	
		Frequency	(%)	Frequency	(%)
1	8	6	75	4	50
2	9	9	100	5	56
3	8	6	75	1	13

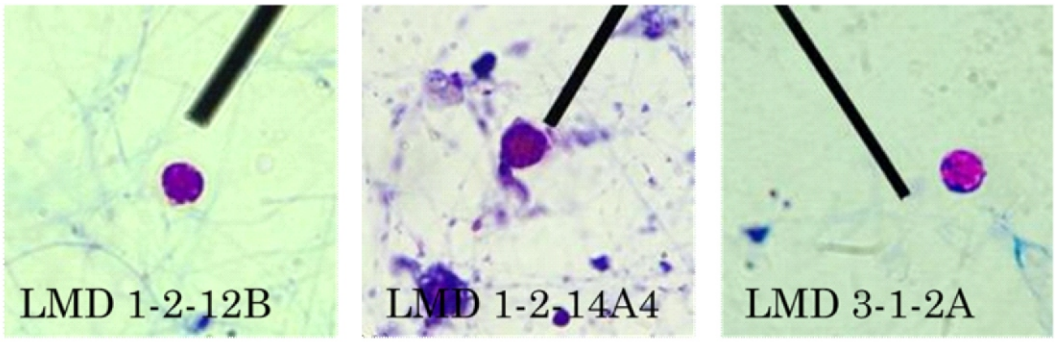

Fig. 4: *Cyclospora* spp. oocyst confirmed by the Research Institute for Tropical Medicine

Cyclospora spp. oocysts, or both. 25 samples from Day 1 showed 17 positive slides for *Cryptosporidium* spp. while 10 were positive for *Cyclospora* spp. giving 68% and 40% positivity respectively. Seventeen samples out of the 25 were found to be positive during the second day; of which, 15 samples (60%) were positive for *Cryptosporidium* spp. oocysts and 8 samples (32%) were positive for *Cyclospora* spp. oocysts. Results of the last day presented with 21 samples (84%) that were positive for *Cryptosporidium* spp. oocysts and 10 samples (40%) were positive for *Cyclospora* spp. oocysts. Table 2. shows that upon comparison, it is clearly seen that higher positivity for *Cryptosporidium* spp. oocysts was observed at 71% (53 out of 75) compared to *Cyclospora* spp. oocyst with only 37% (28 out of 75). Table 3. Breaks down the daily results of positivity per intake eight samples were collected from intakes one and three and nine samples were taken from intake two to have a total of 25 samples per collection day. It should be noted that intake two was closed during the collection dates and that the water there was stagnant and not flowing.Tables 4 to 6. shows daily positivity results for either *Cryptosporidium* spp. oocyst only or *Cyclospora* spp. oocyst only or both in each stained smear preparation and Table 7 is a summary of water samples positive for the target organisms.

All slide preparations were read by licensed medical technologists with training in identification of parasites of public health importance and were sent to the Parasitology Department of the Research Institute for Tropical Medicine for final verification and confirmation. Figure 3 and 4 presents some examples of stained smears positive for the target organisms; Figure 3. shows *Cryptosporidium* spp. oocyst isolated from the sample LMD 1-1-2A, LMD1-1-2B and LMD 1-1-2B2 observed under the oil immersion objective (OIO) and Figure 4. shows

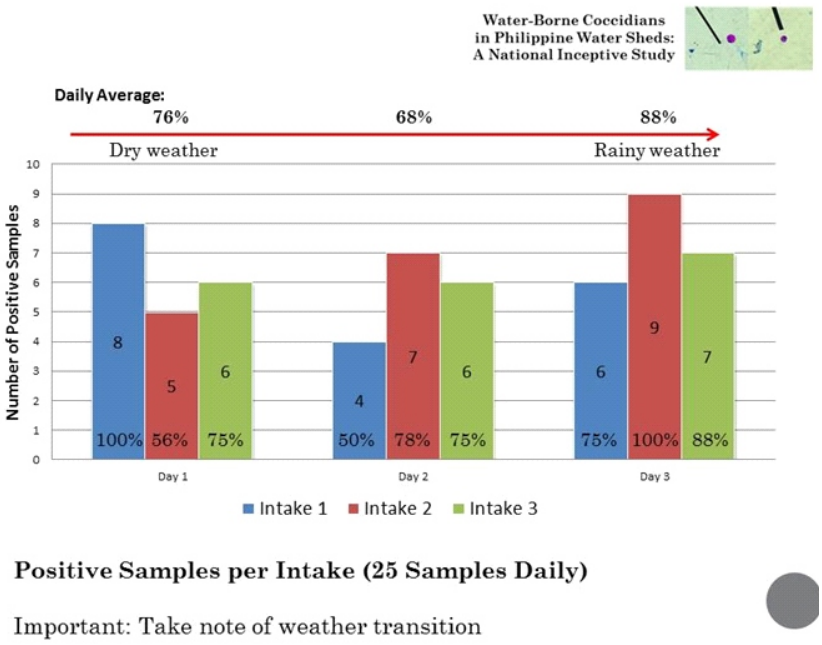

Fig. 5: Variable to increased incidence of target organism during rainfall events and consistent increase in incidence of target organism in stagnant intake (Intake 2)

Table 7. Samples positive for target organisms			
Day	Daily Samples	Positive samples	(%)
Day 1	25	19	76
Day 2	25	17	68
Day 3	25	22	88
Total positive samples		58	77

Table 8. Physicochemical properties of water samples	
Test	**Results**
pH	8.23
Conductivity (uS/cm)	118.8
Turbidity (NTU)	0.913
Total Dissolved Solids (mg/L)	106
Fluoride F^- (mg/L)	Not detected
Chloride Cl^- (mg/L)	2.50
Nitrate NO_3^- (mg/L)	Not detected
Water samples were analyzed at 25 ^{0} Centigrade	

Cyclospora spp. oocyst isolated from the sample LMD 1-2-12B, LMD 1-2-14A4 and LMD3-1-2A observed under OIO. Staining Characteristics, oocyst morphology and size relativity was used to identify *Cryptosporidium* spp. oocysts and differentiate it from *Cyclospora* spp. oocysts that were present. *Cryptosporidium* spp. oocysts are smaller than *Cyclospora* spp. oocyst with average measurements of 4 to 6 and 8 to 10 microns respectively.

Physicochemical Properties of Water Sample

Water samples were chemically analyzed at the Department of Science and Technology, Industrial Technology Development Institute. Table 8. shows the results of the physicochemical analysis of the water samples at 25^0 C. pH was 8.23, Conductivity at 118.8 uS/cm, Turbidity was 0.913 NTU, Total Dissolved Solids at 106 mg/L, Chloride (Cl^-) was 2.50 mg/L and Fluoride (F^-) and Nitrate ($NO3^-$) in mg/L were not detected from the water samples.

DISCUSSION

Observation of the daily results of water samples showed that Day 3 had the highest positivity for *Cryptosporidium* spp. and *Cyclospora* spp. oocyst as compared to Day 1 and Day 2 collections. Day 3 samples were collected after several days of rainfall (Figure 5.); studies indicate that outbreaks caused by drinking-water have been attributed to contamination of the water source by heavy rainfall or snowmelt [16], sewage contamination of wells and inadequate water treatment are potential causes of the same as well [17, 18]. In river water, Cryptosporidium oocyst significantly increased during rainfall events [19] and that the effect

of rainfall on parasite concentrations was due in part to surface runoff, together with the resuspension of river bottom and storm drain sediment [20, 21]. These were contributory factors to the increase in oocyst positivity for Day 3 water samples as the site of collection is a watershed and ecological park surrounded mainly by forests. Climatic conditions can influence the fate and transport of pathogens, as well as their viability, infectivity, stability, and reproduction rates in the environment. Cryptosporidiosis is strongly associated with maximum river flow and a positive correlation between rainfalls [22]. In addition, a wide variety of infected animals and human settlements coming in contact with the La Mesa watershed and its other tributaries like Angat Dam and Ipo Dam which collects its water from the Sierra Madre Mountain Range; may have contributed to the presence of the target organisms in the sampling site; possibly making it positive not only *Cryptosporidium parvum* and *Cyclospora cayetanensis* both of which are coccidians of human health importance but of a zoonotic type of Cryptosporidium as well which is also able to establish itself in human hosts (*Cryptosporidium ubiquitum*) [23].

Some parameters of the physicochemical properties of water have been established to have a positive correlation with oocyst presence, viability and infectivity and are significant parameters to monitor in the study of water-borne protozoans. pH of water samples measured at 25^0 C gave a reading of 8.23; water pH of 6.5 to 8.5 are optimal for viability and survival of Coccidian oocyst; pH less than 4 or greater than 11 results in minor viability loss [24]. The Background Document for Development of WHO Guidelines for Drinking-water Quality [25] sets a standard that water pH of greater than 8.0 may render chlorination ineffective and this may prove very useful in further studies of coccidian profile in water coming out of treatment facilities. Conductivity and Salinity may not be useful predictors of oocyst in surface waters [26], however several studies have demonstrated isolation of *Cryptosporidium parvum* from oysters [27-29] indicating that oocysts are present in brackish marine environments. Chloride is one of the major inorganic anions in saltwater and freshwater which comes from the dissociation of salts, such as sodium chloride or calcium chloride in water; levels were detected at 2.5 mg/L. Oocysts are resistant to chlorine and monochloramine [30]; this issue has translated to recreational-water sources such as swimming pools as potential reservoirs for Coccidian oocysts. Although animal models were able to demonstrate impeded development of *Eimeria magna* oocyst in rabbit by the application of 3.7 X 10-4 M Mercuric chloride [31] such combination and concentration is impractical for human applications. Fluoride and Nitrate were not detected from the water samples; some studies that delved into the influence of these analytes were by using 1.4 mM Phenylmethylsulfonyl fluoride (PMSF) which reduced invasion of *Eimeria tenella* in cultured chicken kidney cells [32] and establishing that increase in cell density increases phosphate and nitrate removal [33]. Additionally, it was found that *C. parvum* oocysts could maintain high levels of infectivity for periods of at least 24 weeks [34] and in water the persistence of oocysts was not affected by temperatures less than 30^0 C [35]; it has even been demonstrated that *C. parvum* oocyst can survive in temperature extremes of 67.5^0 C for 1 minute and at -20^0C for up to 5 hours [36-38]. A variety of chemical and mechanical means may be employed to be able to effectively inactivate, destroy or separate Coccidian oocysts from drinking and recreational water sources. Sedimentation and Filtration provide an effective barrier for Coccidian oocyst and utilizing membrane filtration can further improve the quality of drinking water [39].

CONCLUSION

It has been established for the very First time that La Mesa Dam, a major watershed in Metro Manila, Philippines harbors *Cryptosporidium* spp. and *Cyclospora* spp. oocyst and that isolation of these organisms were more pronounced following rainfall events. Other contributory factors may possibly come from contamination of the watershed itself and other of its tributaries with fecal matter coming from infected animals and human carriers. The pH of the watershed is not optimal for chlorination which can further contribute to the viability and infectivity of the oocysts present and is a significant determinant in its effective transmission to a viable host. Climatic factors, physicochemical characteristics of water sources, the incidence of infection in animal and human populations, as well as the excretion of oocysts in certain watersheds all influence the occurrence of cryptosporidiosis and may very well contribute to Cyclospora outbreak as well. The identification of coccidians of health importance should be considered as an inclusion in the panel of tests in ensuring the potability of water and its cleanliness for household, recreational and agricultural use. The presence of Coccidian oocyst can be used as a means to monitor effectivity of chemical and mechanical methods of treatment of water used for human consumption and various daily activities.

Conflict of interest statement

We declare that there is no conflict of interest.

Ethics Statement

The Researchers provided all the necessary documentations required by the La Mesa Watershed and Eco Park Management and provided the requested data and results at the conclusion of the study.

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