DETECTION AND CHARACTERIZATION OF PROTOZOAN PATHOGENS IN IRRIGATION WATERS USING WATER AND BIOFILM SAMPLING METHODS

by

KATHERINE ANNE WAKELEY

(Under the Direction of Ynés R. Ortega)

ABSTRACT

Irrigation water samples were analyzed for the presence of *C. cayetanensis, Cryptosporidium* spp., and *G. duodenalis*. No association was found between parasite detection and climate (avg. temperature and rainfall). There were six total sampling locations on three different rivers. Locations 3 and 6, were associated with increased *Giardia* and *Cyclospora*-like collection. *Giardia* detection was associated with Autumn (October-December); *Cyclospora*-like and *Cryptosporidium* spp. detection was associated with Spring (April-June). Water and biofilm sampling were not significantly different from each other. Comparisons of filter to swab, filter to brush, and brush to swab yielded no difference in parasite collection between tools. Biofilm sampling can replace water sampling for parasite detection regardless of tool utilized. MLST did not amplify *Cyclospora* positive environmental samples. Sampling for *Cyclospora* and *Cryptosporidium* spp. should occur during Spring when clinical cases of *Cyclospora* and calving season are high, particularly for produce farms downstream of cities or animal farms.

INDEX WORDS *Cryptosporidium*, *Giardia duodenalis, Cyclospora cayetanensis,* environmental sampling, irrigation water

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B.S., The University of Georgia, 2016

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfilment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GA

2018

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August 2018

DEDICATION

This is dedicated to the memory of my grandfather Lewis Banks Sr. (1922-2014).

ACKNOWLEDGEMENTS

I would like to acknowledge Patricia Torres, Alison Payton, and Alexandra Futral for helping me troubleshoot aspects of my thesis and for providing technical support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Cyclospora cayetanensis, Cryptosporidium spp., and *Giardia duodenalis* are parasites of public health importance. Each parasite has been implicated in food and water borne outbreaks across the United States *(1, 3, 15, 19, 23)* and globally *(2, 6, 20, 28)*. Humans are the only known host for *Cyclospora*, while *Giardia* and *Cryptosporidium* spp. have a varied host range including domestic and wild animals. EPA method 1623 *(55)* is the primary method currently used for sampling surface waters for protozoan parasites. This method was originally developed for drinking waters and requires expensive materials and skilled preparation *(9)*. The following thesis seeks to compare the water sampling method, filtration, with two biofilm sampling methods, toothbrush and swab, in an effort to reduce the cost of sampling. The protozoan parasites attach to biofilms in riverine systems *(81, 87, 103, 157)*. Therefore, biofilms can act as a net that sequesters the parasites after a point or non-point source contamination event and prevents them from diluting in the waters. Biofilms are an underutilized river media that can aid public health traceback investigations and farmers identify the protozoan pathogens in the target surface irrigation waters.

The 500 bp nested PCR *(194)* available to the amplify *Cyclospora* DNA is not specific to the *Cyclospora* species and will amplify *Eimeria* spp. and *Isospora* spp. as well. Further characterization of the positive *Cyclospora*-like isolates is required to know if these isolates are

Cyclospora cayetanensis or another Eimerideae. PCR-RFLP *(92)* and sequencing of 500bp PCR are necessary to further characterize the species amplified. *Cyclospora cayetanensis* is an understudied pathogen. Guo et al. *(77)* have recently developed a multilocus sequence typing (MLST) tool to subtype clinical *Cyclospora* samples. These loci successfully subtyped global isolates and determined that clinical isolates from China were genetically distinct from clinical isolates from the United States and Peru *(76)*. With this tool, the confirmed *Cyclospora cayetanensis* positives will be tested to determine if the MLST loci are sufficient to amplify *Cyclospora* in an environmental sample.

Cyclospora cayetanensis

In 1979, R. W. Ashford wrote about a novel coccidian parasite found in the stools of 3 patients in Papua New Guinea *(12)*. Ortega et al. *(134)* later classified this organism as an Apicomplexan similar to the genus *Eimeria* spp. and named it *Cyclospora cayetanensis. Cyclospora* has round oocysts between 8-10 µm in length *(117)*. Humans are the only known host for *Cyclospora cayetanensis* as attempts to experimentally infect mammals and birds were unsuccessful *(50)*. Other species of *Cyclospora* found in non-human primates show similar host specificity *(49)*.

Human clinical symptoms of *Cyclospora* infections include nausea, bloating, weight loss, abdominal discomfort, diarrhea lasting between 1 and 5 weeks, flatulence, abdominal cramping, low grade fever, and fatigue *(135, 137, 138)*. Changes in the small intestine can include shortening and widening of villi, an increase of lymphocyte infiltration at the tip of the shortened villi, and inflammation of lamina propria *(135)*. Circumstantial evidence points to Guillain-Barré *(149)* and Reiter syndrome *(37)* as possible severe sequalae of *Cyclospora* infections in

immunocompetent individuals. Immunocompromised individuals can have a longer illness with more severe symptoms. HIV/AIDS patients were seen to lose more weight than non-HIV/AIDS patients as infection tends to be prolonged *(137)*. *Cyclospora* infection of the gallbladder lead to acalculous cholecystitis in an HIV-positive man *(41)*. Treatment for a *Cyclospora* infection in two patients with AIDS was seen to resolve concurrent biliary disease suggesting that *Cyclospora* can cause biliary disease in AIDS patients *(160)*. Trimethoprim-sulfamethoxazole is currently the only effective treatment for cyclosporiasis in the immunocompromised and immunocompetent *(11)*. Recovery without treatment is possible for those who are allergic to the treatment. Ciprofloxacin has been suggested as an alternative for these patients. However, illness will last several days longer and may result in relapse *(11)*.

Humans contract *Cyclospora* through ingesting food or water contaminated with sporulated oocysts. Person-to-person transmission of *Cyclospora* is unlikely as oocysts are not immediately infective when excreted from an infected host and requires a maturation period lasting days to weeks in the environment to become infective *(83)*. Environmental factors affect the rate of oocyst sporulation. Few studies have been performed to determine optimal environmental factors for oocyst sporulation. Benchtop experiments have been performed to determine *Cyclospora* sporulation at storage, room, and extreme temperatures. Smith et al. *(162)* found that human *Cyclospora*-isolates sporulated after 8 days when stored at 22° and 30°C in potassium dichromate or deionized water. Approximately 15-20% of the oocysts sporulated at these temperatures. Sporulation rates were 0% and 5% at storage temperatures of 4°C and 37°C, respectively, when incubated for 2 weeks *(162)*. Sporulation in dairy, distilled water, and basil at room-temperature (23°) and refrigerator temperatures (4°C) were sufficient to induce sporulation in 50-60% of oocysts in the tested dairy products (milk, diluted milk, and whipped cream) and

30-50% of oocysts in water and basil after 60 min of storage *(155)*. Sporulation was not seen in distilled water or basil after 60 min and 4 days when stored at -20°C or -70°C, respectively.

Once inside the body, sporulated oocysts differentiate into sporozoites, which excyst and infect the epithelial cells lining the duodenum and jejunum of the small intestine *(18, 137, 171)*. Continual reinfection of the host's epithelial cells is facilitated through asexual reproduction when the sporozoites mature into Type I then Type II merozoites *(137)*. Unsporulated oocysts are developed through sexual reproduction in which Type II merozoites differentiate into microgametocytes and macrogametocytes. The former fertilizes the latter to form a zygote that eventually becomes the oocyst *(137)*. The cycle renews when the unsporulated oocyst is excreted into the environment.

***Cryptosporidium* spp.**

Cryptosporidium spp. was originally observed by Edward Tyzzer in 1907 in the intestines of lab mice. The microorganism was considered a commensal organism until 1976 when reports of cryptosporidiosis in humans first occurred *(180)*. *C. parvum* and *C. hominis* are the most common species to cause cryptosporidiosis in humans though other zoonotic *Cryptosporidium* spp. have been linked to human infections. Table 1.1 describes the other species and their primary hosts in further detail.

Table 1.1. *Cryptosporidium* species implicated in previous outbreaks adapted from Xiao and Feng *(191)*.

Species	**Primary Host**
C. parvum	Ruminants
C. hominis	Humans, horses
C. suis	Swine
C. felis	Cats
C. canis	Dogs
C. meleagridis	Galliformes
C. muris	Rodents
C. andersoni	Cattle

Symptoms of cryptosporidiosis include self-limiting, watery diarrhea, vomiting, nausea, fever, and abdominal cramps that last about 2 weeks *(111)* in the immunocompetent. Nitaxozanide is an approved treatment for *Cryptosporidium* spp. infections. Cryptosporidiosis symptoms can vary in severity depending on the immune status of the infected and can last up to 30 days in the immunocompromised *(106)*. After contracting cryptosporidiosis from contaminated recreational waters, symptoms resolved for a seven year old renal transplant patient 22 days after hospitalization *(89)*. *Cryptosporidium* spp. infections were significantly associated with HIV/AIDS positive patients with the likelihood of infection increasing as $CD4^+$ T-cell counts decreased *(13, 99)*. Organ transplant patients are susceptible to cryptosporidiosis. Rare incidences of *Cryptosporidium* spp. colonizing the respiratory system *(72, 148)*, joints, liver *(43)*, and biliary duct *(43)* have occurred. Antiretroviral and other therapies that decrease immunosuppression are useful for eliminating *Cryptosporidium* spp. from the immunocompromised *(89, 99)*.

Cryptosporidium spp. transmission can occur person-to-person or zoonotically through the ingestion of contaminated food or water. Once ingested, sporozoites excyst and infect enterocytes of the upper small intestine. Attachment to the enterocytes spurs *Cryptosporidium* spp. to become a type I meront that differentiates into 6-8 merozoites. These merozoites leave the initial attachment site where it can multiple in the host through asexual reproduction or differentiate in to a type II meront to undergo sexual reproduction. Type II meronts produce 4 merozoites that differentiate into micro- and macrogametes within the hosts cells. Upon fertilization of the macrogamete with the microgamete, thin and thick-walled oocysts are produced. Thin-walled oocysts re-infect the host; thick-walled oocysts are excreted by the host into the environment. Unlike *Cyclospora cayetanensis, Cryptosporidium* spp. are immediately infectious upon excretion *(111)*.

Giardia duodenalis

Giardia was first described by Antonie van Leeuwenhoek in 1681 and later by Vilém Dušan Lambl in 1859 as a commensal organism living in the human intestines. In the 1970's, the first human cases of *Giardia duodenalis* disproved that hypothesis *(4, 115)*. *Giardia duodenalis* Assemblages A and B are the only *Giardia* subtypes known to infect humans. Several *Giardia* species are not infectious to humans. These species include *G. cati* in cats *(142), G. canis* in dogs *(142), G. muris* and *G. microti* in rodents *(29)*, *G. agilis* in amphibians *(29, 164),* and *G. ardeae* and *G. psittaci* in birds *(29)*.

The *Giardia* life cycle begins when a suitable host ingests cysts through contaminated food or water or contact with infected humans or animals. Stomach acid and bile salts stimulate

the cyst to excyst into 2 trophozoites in the upper small intestine. *Giardia* reproduces in the small intestine through binary fission. Each trophozoite has 4 pairs of flagella and a sucking disk that is used to adhere to the lining of the small intestine *(133)*. *In vitro* introduction of bile salts and fatty acids at a slightly alkaline pH was shown to encourage encystation of *Giardia* trophozoites *(74)*.

Giardiasis symptoms include flatulence, abdominal cramps, bloating, nausea, anorexia, weight loss, and, occasionally, fever. Treatment is necessary to reduce the duration of the infection, which can last for weeks to months without proper care *(133)*. Reduced immune capacity has been linked with chronic giardiasis *(125)*. Anorexia and weight loss are symptoms of nutrient malabsorption that occurs during giardiasis. Steatorrhea, abnormal xylose excretion, poor absorption of glucose, decreased disaccharide enzyme activity, and Vitamin A, B12, and folic acid deficiency were observed in patients with confirmed giardiasis *(90, 125)*. One likely explanation for nutrient malabsorption is *Giardia*'s method of attachment. The mechanical attachment of *Giardia* to the intestinal lining may create a barrier between the intestines and nutrients passing through the bowel *(26)*. Nutrient malabsorption maybe due to changes to the architecture of the small intestine like shortening of the microvilli, thickening of mucosal folds, and noticeable inflammatory infiltration of the surrounding tissues *(90)*. One course of metronidazole or one dose of tinidazole are effective for treating giardiasis *(133)* and improving nutrient malabsorption *(90)* in the immunocompetent and the immunocompromised.

Global Distribution

Cyclospora, Cryptosporidium spp. and *Giardia* are globally distributed pathogens. *Cyclospora* has been detected in the waters, produce, or human clinical samples of the United States *(78,*

102), Canada *(45, 46, 109)*, Italy *(73)*, China *(113, 194)*, Mexico *(132)*, Ethiopia *(175)*, Vietnam *(178)*, Malaysia *(22)*, Guatemala *(30)*, Haiti *(116)*, Indonesia *(25)*, Honduras *(95)*, Egypt *(98)*, Peru *(87, 136)*, Nepal *(159)* among other countries and is known to cause foodborne illness in travelers to developing countries *(57, 121, 130, 174)*. MLST subtyping of *Cyclospora* isolates has only recently become available to describe the relationship between global *Cyclospora* isolates. Isolates from Peru and the U.S. were more similar to each other than to isolates from China *(76)*.

Giardia is found globally. Regardless of whether a country is developed or developing, *Giardia* assemblage B is detected more often than assemblage A *(108, 153)*. Countries like Nicaragua *(110)*, Nepal *(161)*, Germany *(27)* among others predominantly detected assemblage B similar to the global trend. Not all countries show the same prevalence rates. Assemblage A is the primary assemblage found in the Middle Eastern and African studies *(108)*. Australian human *(187)* and Chinese wastewater *(113)* samples were primarily assemblage A. Samples from Mexican children had primarily assemblage AI and AII *(54)*. Assemblages A and B were detected in Rio de Janeiro at about the same rate *(58)*.

Cryptosporidium spp. is unique from the other organisms being studied as multiple species of *Cryptosporidium* can infect humans. *C. parvum* and *C. hominis* cause the most *Cryptosporidium* cases globally and will be the species under discussion. European cryptosporidiosis cases were predominantly a mixture of *C. parvum* and *C. hominis* infections, while infections in the Middle East are mostly *C. parvum (190). C. parvum* subtypes IIa and IId and *C. hominis* subtypes Ia, Ib, Id, and Ie are the most common subtype families found worldwide *(68)*. The predominant *C. parvum* subtype families can vary between geographic regions; subtype families IIa, IId, and Ill are common to European calves. These are the primary

subtype families in human infections in areas with large bovine populations *(190)*. Anthroponotic species *C. hominis* and *C. parvum* subtype family IIc are the primary subtypes found in developing countries *(190)*. IIc is considered only anthroponotic and has been found in human isolates from North America, Europe, and Australia *(190)*.

C. hominis infections in developing countries are highly diverse with multiple subtypes detected during sampling periods. Ia, Ib, Id, and Ie were the predominant subtype families found in human fecal samples from Kolkata, India *(70)*. The rare If subtype family along with Ia, Ib, Id, and Ie were found in northern India *(192)*. *C. hominis* subtype families Ia, Ib, Id, and Ie were detected in child fecal samples in Sonora, Mexico. A one-year old female was infected with the novel subtype IaA14R11, which had not been detected anywhere else in the world *(181)*.

Subtype family Ib was the predominant family detected in developed nations *(190)*. Three Ib subtypes (IbA19G2, IbA20G2, and IbA21G2) and one Ie subtype (IeA12G3T3) of *C. hominis* were found in wastewater samples taken in Shanghai, China *(62)*. The same subtypes, once thought to be unique to Shanghai, were found in Shanghai, Nanjing, Qingdao, and Wuhan, China wastewater samples taken a few years later *(113)*. *C. parvum* subtype families IIa and IId and *C. hominis* subtype families Ib and Ig were the predominant families found in human clinical samples and animal samples taken from hospitals, farms, zoos, and urban wildlife in New Zealand *(68)*. Portuguese human *C. hominis* cases were primarily from subtype family Ib (10/15) *(7)*.

Animals

Cyclospora cayetanensis, *Giardia duodenalis*, and *Cryptosporidium* spp. are just a few parasites known to infect humans. These foodborne pathogens can contaminate fresh produce at

the farm level and ending at the consumer. Worker hygiene and irrigation water are two primary pathogen introduction routes at the farm level. Establishing basic hygiene expectations and the use of outer coverings like gloves, hair nets, face masks, etc. is recommended to prevent direct contamination of produce by infected workers *(66, 126)*. Surface waters are vulnerable to point and non-point source pollution and are known reservoirs for the pathogens being studied *(5, 94, 127, 150, 167)*. Surrounding land use by livestock raisers can result in non-point source contamination of waters. Adequate pest control is necessary to prevent contaminating produce in the fields and processing facilities.

Domestic and wild animals can serve as reservoirs for the parasitic protozoa. Farm animals, particularly cattle, are the primary reservoir for *Cryptosporidium parvum*. Age of cattle was not a determinant of *Cryptosporidium* spp. prevalence in cattle in Southern Ghana *(165)*. Calves less than 10 months (25.8%), between 11-24 months (33.3%), and cows greater than 24 months (27.6%) showed no statistically different *Cryptosporidium* prevalence rate *(165)*. Though *Cryptosporidium* spp. prevalence is not dependent on age, various *Cryptosporidium* species are found more readily in cattle at different life stages. For example, *C. parvum* is primarily shed by calves *(163)*. In the eastern United States, pre-weaned calves had higher rates of *C. parvum* than the other life stages. The prevalence of *C. parvum* decreased in older cattle with mature dairy cows having primarily *C. andersoni* (66%) *(61)*. Of the shedding pre-weaned calves in the Nile River delta, Egypt, 43.5% shed *C. parvum*. The other species found were *C. ryanae* (18.8%), *C. bovis* (10.2%), *C. andersoni* (10.2%) *(8)*.

Other domestic ruminants, such as sheep and goats, are known to carry *C. parvum*. Goats and sheep raised in semi-arid and arid conditions were 18.66% and 21.33% positive for *C. parvum,* respectively *(158)*. Nine free-range goat kids in Turkey were found to be infected with 4

different *C. parvum* subtypes, IIaA13G2R1, IIaA15G1R1, IIdA22G1, and IIdA18G1, which are commonly found in humans *(173)*.

Giardia duodenalis Assemblages A and B, known to infect humans, can also infect swine. Data on the prevalence of *Giardia* in domestic or wild pigs and boars is conflicting and may be regionally specific or impacted by raising method. Wild pigs in Texas showed a 4.3% prevalence of *G. duodenalis* of any assemblage *(151)*. *Giardia* Assemblage A was identified in 15.4% of Danish pigs raised under organic farming practices. The remaining 84.6% of *Giardia* samples were characterized as Assemblage E *(143)*. Captive wild boar and domestic pigs in China were positive for *Giardia* 3.1% *(114)* and 8% *(183)*, respectively, but predominantly with Assemblage E, which does not infect humans.

Aquatic rodents are sources of waterborne *Giardia* Assemblages A and B *(47)*. Four beavers in Massachusetts tested positive for *Giardia* Assemblage B by IFA and sequencing of the TPI, ssrRNA, and β-giardin genes *(59)*. Proximity to beaver lodges was directly associated with increasing *Giardia* cyst concentrations *(166)*. A beaver nesting near the water intake of a drinking water plant in British Columbia, Canada tested positive for *Giardia* Assemblage B, the same assemblage found in three outbreak patients in the same area *(179)*. *Giardia* spp. were found in 33% of beavers and 73% of nutria sampled from East Texas *(47)*. Low prevalence of *Giardia duodenalis* Assemblages A and B was found in land rodents in Germany of the genera *Apodemus, Microtus,* and *Myodes* indicating land rodents are not a significant reservoir for *Giardia (82)*.

Other animals that may be potential reservoirs for *G. duodenalis* are primates and ruminants living near areas heavily trafficked by humans. Wild rhesus macaques in North West India living close to humans had a 31% prevalence of *Giardia* Assemblage B, though zoonotic

transmission between humans and primates was not fully determined *(42)*. Gorillas, forest buffalo, and domestic cattle living near tourist attractions tested positive for *Giardia* at a rate of 9%, 2%, and 6%, respectively. The gorillas tested positive for *Giardia* subtypes commonly found in humans suggesting zoonotic transmission of *Giardia* from humans to the gorillas *(88)*. Prevalence of human *Giardia* subtypes in the animals does not prove that transmission of *Giardia* is occurring from humans to the animals. These studies do show that human subtypes may be able to reproduce in non-human primates, domestic and wild ruminants.

Zoonotic transmission of *Cryptosporidium* spp. and *G. duodenalis* can occur between domestic or wild animals and humans. *Cryptosporidium* spp. infections in the U.S. were reported the highest in the Midwest at 45.8% and 40.4% of all cases reported in 2011 and 2012, respectively, likely due to the increased presence and exposure of the residents to cattle *(141)*. Cryptosporidiosis outbreak at a North Carolina summer camp linked handling infected calves and eating produce grown next to the calves as contributing factors toward infection *(36)*. Transmission through animal contact accounted for 1.2% of all *Giardia* outbreaks from 1971-2011 in the U.S. *(3)*. Transmission of *Giardia* cysts between the food preparer and a pet rabbit was a suspected route of transmission for a giardiasis outbreak in 1981. Zoonotic transmission was not confirmed as the species of *Giardia* found in the stool of the rabbit was never specified. Person-to-person transmission was also suspected as the food preparer's toddler tested positive for *Giardia duodenalis* *(145)*.

Unlike *Giardia* and most *Cryptosporidium* spp., humans are the only known host for *Cyclospora cayetanensis.* Suspected *C. cayetanensis* was found in captive chimpanzees and cynomolgus monkeys in Europe. The author reports that not enough information is known about *Cyclospora* spp. to definitively assert that non-human primates are hosts of *C. cayetanensis*

(120). A prior study failed to experimentally infect several types of mammals (dogs, jirds, rabbits, hamsters, ferrets, pigs, sand rats, rats, and 9 types of immunocompetent and immune-deficient adult and neonatal mice), birds (chickens and ducks), and non-human primates (owl, rhesus, and cynomolgus monkeys) with *C. cayetanensis (50)*. This evidence suggests that *C. cayetanensis* infections in non-humans may require host specific adaptations *(120)* or that non-human primates' prolonged contact with infected humans may facilitate infections.

Giardia and *Cryptosporidium* spp. can be transmitted person-to-person. Anthroponotic transmission was considered a secondary route of transmission between infected campers and staff at the North Carolina summer camp *(36)*. *C. hominis*, whose primary host is humans, was found in 89.3% of Egyptian schoolchildren presenting gastrointestinal illness suggesting anthroponotic transmission *(52)*. Anthroponotic transmission is the implied transmission route for English and Welsh *C. hominis* infections because they were closely associated with living near another person with diarrhea and living in a city *(34)*. Adam et al. *(3)* found that 2.5% of giardiasis outbreaks between 1971-2011 were the result of person-to-person transmission, which occurred primarily in households and at daycares *(3, 23, 140, 145, 168)*. Secondary transmission of *Giardia* from sickened school employees to their family members was confirmed in Minnesota by a public health inquiry *(139)*. Person-to-person transmission of *Cyclospora* is unlikely as *Cyclospora* oocysts requires a two-week period to mature and become infectious.

Food

Consumers can be introduced to *Cryptosporidium* spp.*, Cyclospora,* and *Giardia duodenalis* through contaminated food and water. The prevalence of protozoan parasites on fresh produce varies from country to country. *Cryptosporidium* oocysts were found in 5.9% of Canadian leafy

greens *(46)*, 6% of Indian vegetables (turnip, cabbage, carrot, chili, coriander, cucumber, radishes, and tomatoes) *(182)*, and 35% of all vegetables sampled in Nigeria *(119)*. Utaaker et al. *(182)* found that vegetables sold in supermarkets were more contaminated with *Cryptosporidium* than vegetables sold in open-air markets. Vegetables grown near high densities of cattle in Poland were contaminated with *Cryptosporidium* spp. oocysts *(154)*. *Cyclospora* was detected on 12.2% of cucumber, lettuce, celery, fennel, melon, tomato, endive, melon and chicory samples taken from farms in southern Italy *(73)*. Six and one of 1,171 packaged leafy green samples from Canada were positive for *Cyclospora* and *C. parvum*, respectively *(109)*. Fruits and vegetables sampled from 4 markets in southern Ethiopia contained *Cryptosporidium* spp. (4.72%), *G. duodenalis* (10.0%), and *Cyclospora* spp. (6.94%) *(16)*. In southwestern Ethiopia, 12.8%, 7.5%, and 5.0% of the vegetables from markets were contaminated with *Cryptosporidium* spp., *G. duodenalis*, and *Cyclospora* spp., respectively *(175)*. *Cryptosporidium* spp. (29.3%), *Giardia* spp. (6.7%), and *Cyclospora* spp. (21.3%) were found on raw vegetables collected from Alexandria, Egypt *(53)*. A Canadian study detected *Cyclospora* (1.7%), *Cryptosporidium* spp. (5.9%), and *Giardia* (1.8%) on RTE leafy greens purchased from common retailers *(46)*. The main limitation of these studies is that they do not assess infectivity of the oocysts found. Environmental factors stressing the oocysts may inactivate them before consumption by the consumer. Another limitation is that prevalence of oocysts in pre-consumer samples do not account for consumer food hygiene habits.

Cyclospora outbreaks are highly associated with foodborne transmission. An estimated 99% of domestically acquired *Cyclospora* infections in the U.S. were connected to contaminated foods *(156)* with imported fresh berries, sugar peas, snow peas, fresh herbs, and salad greens being the most commonly implicated foods *(31)*. Between 1995 and 2000, several foodborne

outbreaks were caused by raspberries imported from Guatemala *(30)* including outbreaks in Boston *(65)* and Philadelphia *(86)*. Outbreaks of cyclosporiasis from 2013 to 2015 implicated imported salad greens and cilantro originating from Mexico *(31)*. As a result of the findings of these outbreaks, an U.S. FDA import alert *(67)* is in effect for cilantro imported from Puebla, Mexico *(1, 31)*. A recent outbreak of cyclosporiasis in the United States affected consumers of a fresh vegetable tray *(32)*.

About 8% of domestically acquired US cryptosporidiosis outbreaks were linked to foodborne transmission *(140)*. Ozonated apple cider was the cause of a 2003 cryptosporidiosis in Ohio *(24)*. An outbreak occurred in 1993 at a Maine agricultural fair when apples contaminated with calf feces was used to make the cider *(123)*. In 1996, concurrent outbreaks in New York and Connecticut were attributed to different steps in fresh pressed cider processing. The Connecticut outbreak was clearly linked to their practice of using apples that had fallen to the ground (i.e. drop apples) in their cider. No clear source of contamination was found in the New York outbreak; all samples tested negative for *Cryptosporidium* spp. *(10)*. *Cryptosporidium* outbreaks have also been linked to food worldwide. Frisé salads were implicated in 5 Finnish cryptosporidiosis outbreaks occurring in 2012 *(2)*.

Foodborne transmission of *Giardia* is less common that for *Cyclospora* and *Cryptosporidium* spp. Less than 1% of domestically acquired U.S. giardiasis outbreaks were linked to contaminated food between 2011-2012 *(140)*. While rare, foodborne outbreaks do still occur with 15.7% of giardiasis outbreaks since 1971-2011 attributed to food *(3)*. Twenty-nine employees of a Minnesota school district were sickened from a home-canned salmon dish contaminated with *Giardia* in 1979. The salmon dish was likely contaminated by the preparer as she had previously diapered her asymptomatic grandson *(139)*. Poor hygiene was noted as the

cause for *Giardia* contamination of fruit salad resulting in 10 cases of giardiasis in New Jersey in 1986 *(145)*. Noodle salad was implicated as the most likely cause of a giardiasis outbreak in western Connecticut as the salad preparer and her children tested positive for *Giardia* oocyst with stool analysis. The preparer's symptoms occurred within one day of serving the salad indicating that she was infected before the initial event *(144)*. A 2015 giardiasis outbreak on Long Island resulted in 20 cases linked to asymptomatic employees preparing RTE foods and produce. The three infected employees tested positive for *Giardia* assemblage BIII, which was also found in 2 of the lab-confirmed cases *(64)*.

Wastewater Treatment

Untreated human wastes can contaminate surface and drinking waters with pathogenic protozoan. Raw wastewater samples from Shanghai, China contained primarily *C. meleagridis* (11.1%) and the novel *C. hominis* (93.7%) subtypes, Ib19G2, Ib20G2, and Ib21G2 *(62)*. *Cryptosporidium* spp. (56.2%), *Giardia* (82.6%), and *Cyclospora*-like organisms (80.3%) were detected in sewage samples taken from Shanghai, Nanjing, Wuhan, and Qingdao, China *(113)*. Wastewater treatment facilities are designed to remove or inactivate pathogens to prevent releasing contaminated waters into the environment. Filtration, sedimentation, flocculation, and coagulation are treatment processes with the potential to partially remove *Cryptosporidium* spp., *Cyclospora,* and *Giardia* from water *(21, 56, 129, 137)*. UV radiation, ozonation, and chlorination are disinfection methods that have varying degrees of success against protozoan parasites. *C. parvum* oocyst inactivation could not be achieved under practical exposure times with chlorine based disinfectants *(105)*. However, medium-pressure UV radiation at a level of 60mJ/cm^2 or more permanently inactivated *Cryptosporidium parvum* and *Giardia muris*

[oo]cysts *(17)*. Combining disinfection methods can improve pathogen inactivation. Water turbidity can reduce UV irradiation efficacy. Ozone pretreatment of unfiltered drinking water (0-20NTU) was found to have a synergistic effect with UV irradiation to inactivate *Giardia* and *Cryptosporidium parvum (79)*.

Environmental Persistence

Pathogens that are not contained through wastewater treatment measures are susceptible to harsh environmental conditions. *Cryptosporidium* spp. and *Giardia* susceptibility to environmental stressors have been written about extensively. *Giardia* was found to be more susceptible to sunlight in marine environments *(93)* and temperature extremes *(131)* than *Cryptosporidium* spp. Sunlight and high salt concentrations were sufficient to inactivate *Giardia muris* by 3 $\log_{10}$ in 3h in marine waters *(93)*. The same study found *Cryptosporidium* spp. required 3-4 days to achieve a 1-log reduction of oocyst viability *(93)*. Water turbidity can increase the survival of *C. parvum* in high UV conditions (900W/m^2) *(75)*. *G. muris* cysts were inactivated at -4°C and 25°C at 1 and 2 weeks, respectively *(131)*. King et al. *(100)* showed that *Cryptosporidium* spp. oocyst inactivation at 20°C and 25°C was correlated to ATP depletion in the oocyst. *Cryptosporidium* spp. oocysts frozen at temperatures below -15°C for greater than 168h and -20°C for 24h were not infectious to neonatal mice *(60)*. Little is known about the susceptibility of *Cyclospora* to environmental conditions. *Cyclospora* is thought to prefer moist environments for sporulation *(169)*. Further research is needed to fully assess the environmental persistence of *Cyclospora* in river systems.

Biofilms can act as a net that catch contaminants as they pass through river systems. A biofilm's life cycle of growth, detachment, and regrowth provide opportunities for planktonic

pathogens to attach to biofilms and for these pathogens to be later released into the environment *(112)*. Biofilm sampling of a Peruvian river system collected *Giardia duodenalis, Cyclospora cayetanensis,* and *Cryptosporidium* spp. in 26.4%, 2.2%, and 2.2% (n=178) by nested PCR, respectively *(87)*. *Giardia duodenalis* and *Cryptosporidium parvum* [oo]cysts are seen to attach to established biofilms in drinking water distribution systems within an hour of their introduction and detach with changing flow conditions *(81)*. *Cryptosporidium* spp. oocysts were seen to excyst *(104)* and multiply *(103)* within a laboratory created *Pseudomonas aeruginosa* biofilm. Searcy et al. *(157)* found that *C. parvum* oocysts were conserved in *Pseudomonas aeruginosa* biofilms for longer than 24 hours at high flow conditions.

Drinking and Recreational Waters

Human exposure to protozoans can occur through direct contact with contaminated drinking and recreational waters. Proper drinking water purification is necessary to prevent municipal dissemination of the parasites through treated drinking water systems. Influent samples from the Blankaart catchment in Belgium were 92% and 96% positive for *Giardia* and *Cryptosporidium* spp., respectively. Protozoan parasites were not detected in treated tap water exiting the catchment (22). *Cryptosporidium* spp. and *Giardia* [oo]cysts were detected in 100% and 92% of water samples, respectively, entering a Japanese water purification plant on the Sagami River *(80)*. *Cryptosporidium* spp. and *Giardia* oocysts were detected in the influent of 20 drinking water facilities in Northeastern Spain. Eleven of the 20 plants had effluent positive for protozoa and the type of filtration (rapid filtration, sand plus activated carbon filtering, or slow filtration) did not seem to play a difference in [oo]cyst recovery in effluent *(147)*. Effluent samples for 25 drinking water facilities in the United States detected *Cryptosporidium* spp. and

Giardia in 25 and 46% of the samples *(101)*. Municipal treatment centers drawing on surface waters are not the only water sources vulnerable to contamination. Higher risk of *Cryptosporidium* spp. infections was associated with residents in New Mexico who had one-site waste management systems and utilized well water *(177)*.

Cryptosporidium spp. and *Giardia* outbreaks accounted for 29% of drinking water outbreaks in the U.S. between 2013 and 2014 *(19)*. Drinking water was the leading cause of giardiasis waterborne outbreaks with 74.6% of the outbreaks attributed to drinking water from 1971 to 2006 *(3)*. Furthermore, 86% of 123 drinking water outbreaks caused by a parasite were due to *Giardia.* Drinking from a roadside spring caused an outbreak in New York State and Massachusetts residents *(15)*. A New Hampshire outbreak was associated with drinking tap water sourced from a well that was drilled too close to surface waters and unapproved by the NHDES. The well was considered to be likely contaminated by nearby surface waters due to high fecal-coliforms and the presence of diatoms, algae, rotifers, insects and larvae, and other debris *(40)*. An outbreak in Montana *(185)* was caused by inadequately processed municipal drinking water system. The systems did not filter or chlorinate the water before distribution and heavy water runoff was speculated to be the cause of the contamination *(185)*. *Cyclospora* oocysts were found in 59.3% of drinking water sachets in Accra, Ghana; *Cryptosporidium* spp. oocysts were found in 63.0% of the sachets *(107)*.

Cryptosporidiosis outbreaks in Milwaukee *(118)*, Las Vegas *(152)*, and Oregon *(44)* were linked to drinking municipal tap water in 1993, 1994, and 2013, respectively. Contamination of effluent waters by runoff from nearby cattle pastures was thought to be the initial source of contamination in Milwaukee. Speciation of Milwaukee samples identified *C. hominis* as the causative agent ruling out cattle contamination as the cause *(170)*. The public health

investigation of the Las Vegas outbreak yielded no definitive source of the outbreak. Furthermore, the outbreak onset was linked to a facility with no treatment deficiencies and resolved on its own with no changes being made to the quality of influent water at the facility *(152)*. A 2013 drinking water outbreak in Oregon was caused by contamination of the source water with cow feces. The facility was not equipped to remove *Cryptosporidium* spp. oocysts from their waters as chlorination was the primary disinfection step and filtering had yet to be implemented *(44)*.

In 1990, *Cyclospora* was implicated in an outbreak associated with drinking water at a hospital in Chicago, IL *(39)*. A public health investigation of the outbreak could not pinpoint the source of the outbreak. However, stagnant water in a storage tank supplying the physician's dormitory with water was considered the cause *(91)*. In 1994, British soldiers in Nepal were sickened by *Cyclospora* from their drinking water supply. The implemented filtration step was not sufficient to remove *Cyclospora* oocysts from the source waters and the chlorination step was not sufficient to kill the organism *(146)*. An outbreak of *Cyclospora* and *Cryptosporidium* spp. in western Turkey resulted in nine percent of the population experiencing a co-infection of *Cyclospora* and *Cryptosporidium* spp. Fifty nine percent were infected with only *Cryptosporidium* spp. and 31.8% were infected with only *Cyclospora.* The communal drinking water tank was the suspected source of the outbreak. However, *Cyclospora* oocysts were not detected in samples taken from the tank *(6)*.

As the protozoan parasites can be transmitted through water, recreational water use is another possible transmission route. *Cryptosporidium* spp. is the least susceptible parasite to halogenation, the most common treatment for recreational water, and is the primary cause of outbreaks associated with recreational waters *(85)*. In 2011-2012, *Cryptosporidium* spp. caused

52% of infections associated with treated recreational waters in the United States *(85)*. An estimated 336 people were sickened at a community theme park in California after employees positive for diarrhea continued to work in the pools *(48)*. An outbreak of *C. hominis* in Kansas was associated with swimming pools and day-care centers that used recreational waters *(48)*. Inadequate sanitation of recycled waters at a New York splash pad resulted in 2,307 people developing cryptosporidiosis *(193)*. These outbreaks highlight the need for complete sanitation of community recreational waters to prevent the spread of *Cryptosporidium* spp. to susceptible hosts.

Giardia infections from recreational waters are less common. *Giardia* caused 3 outbreaks between 2011-2012 *(85)*. Giardia was found in 7 lakes, 2 swimming pools, 2 splash parks, and 3 water fountains used for recreational purposes in Belgium *(51)*. Contact with the children's pool was strongly associated with a giardiasis outbreak in Boston, Massachusetts *(96)*. One incidence of *Cyclospora* infection due to recreational water exposure occurred. Organisms that were about 8-9 µm in length were found in the stool of a 6-year-old boy. The boy reported swimming in Lake Michigan 1 day before diarrhea presented itself and tested negative for *Salmonella, Shigella,* and *Campylobacter (189).*

Environmental Control

Environmental control of parasitic pathogens begins with understanding their host range and implementing steps to a) prevent host infection and b) prevent the spread of the parasite to other hosts and into the environment. Improved sanitation, clean water and food, and an emphasis on disinfection of contaminated materials can reduce the numbers of [oo]cysts released into the environment. Few chemical sanitizers can effectively inactivate *Giardia* and C*ryptosporidium*

spp. [oo]cysts. Hand sanitizers with ethanol or isopropanol reduced *Giardia* cysts concentrations by 85-100% and their infectivity *(35)*. Undiluted 4% acetic acid decreased the viability of *Giardia* cysts to zero after 1 hour at room temperature. A 1:1 ratio of acetic acid to water reduced the viability of the cyst to 1.8% in the same conditions *(38)*. Hydrogen peroxide at 6 and 7.5% proved an effective disinfectant for endoscopes. Lower hydrogen peroxide concentrations, ammonia, and bleach did not successfully reduce *Cryptosporidium* spp. concentrations *(14)*. Removal of calf bedding, feces, and using hot water or steam to clean contaminated facilities, and using hydrogen peroxide as a sanitizer are suggested control methods for calving facilities *(176)*. Utilizing the effective chemical sanitizers can reduce transmission of protozoan intestinal illness between organisms and surfaces.

Adequate human waste management is a mitigation method employed by governments and individuals through centralized and on-site treatment facilities to prevent point source pollution of human wastes. Land application of manure and waste from animal agriculture are significant sources of fecal contamination for surface waterways *(84)*. Prevention of non-point source pollution is accomplished through ecological interventions. Reducing cattle grazing access to riparian zones is shown to improve water quality *(172)*. Vegetated buffers and straw mulch placed near cattle were seen to reduce the concentration of *Cryptosporidium* spp. oocysts in storm water runoff *(124)*. Matthaei et al. *(188)* discovered that native New Zealand grasses were sufficient to reduce *Giardia* concentrations in runoff in riparian zones. Ecological interventions have the potential to greatly reduce the concentrations of [oo]cysts entering waterways when applied effectively.

Travel-related Infections

Countries with poor food and water infrastructure or a breakdown in public health measures pose a unique challenge to travelers. Individuals travelling abroad can contract the protozoan parasites through contaminated food or water. An estimated 9%, 42%, and 8% of *Cryptosporidium* spp., *Cyclospora,* and *Giardia* infections, respectively, in the United States were travel-related *(156)*. Between 2004-2009, U.S. travel-related *Cyclospora* and *Cryptosporidium* infections were attributed to travel to Latin America *(97)*.

Traveling to developing nations has also caused travel-related illnesses in Canada and British Columbia. A recent spike in *Cyclospora* infections in British citizens was attributed to travel to Mexico *(57)*. A Riviera Maya (Mexico) outbreak resulted in 79 British and 97 Canadian cases of cyclosporiasis *(130)*. Much of travel-related illnesses reported in British Columbia were associated with travel to Asia (40%) and Mexico (23.1%). *Cyclospora* infections were attributed to travel to South America *(174)*.*Cryptosporidium* spp. caused diarrhea in 2.9% of patients at a clinic in Berlin, Germany *(184)*. Six percent (14/281) of travelers returning to North America from Mexico had *Cryptosporidium* spp. infections. *C. parvum* (13/14) caused most of those infections *(128)*. British patients, who had recently travelled, more commonly had *C. hominis* than *C. parvum* according to isolates taken in 2000 to 2003 *(33)*. *Giardia* assemblages A and B were detected in traveler's returning to Germany *(27)*. *Giardia* was the cause of 9% of the diarrheal diseases seen in patients returning home from Brazil between 1997-2013 *(186)*. *Giardia* was the most common cause of diarrhea among patients visiting EuroTravNet clinics and comprised 16% of the diarrhea patients *(71)*. Accurate pre-travel education can reduce the need for medical care abroad and upon return *(122)*.

Multilocus Sequence Typing (MLST)

MLST is a molecular characterization tool used to subtype species. Traditionally, the GP60 locus was the primary gene used to subtype *Cryptosporidium* spp. Additional genes are utilized for MLST subtyping to further differentiate subtypes and to understand the population changes that occur due to geographic isolation and recombination events. Feng et al. *(63)* was able to subtype clinical isolates from Texas, New Mexico, Arizona, and Ohio using the GP60, CP47, CP56, DZ-HRGP, HSP70, MSC6-7, Mucin1, and RPGR loci and show a link between geography and MLG groups. The subtype IaA28R4, which is becoming a major subtype in the United States, had two distinct MLG groups, one from Ohio and the other from the southwestern states. GP60 subtypes in India *(70)* and Jamaica *(69)* were considered distinct from *C. hominis* subtypes in other developing nations based on their MLST groups *(191)*. The distinction between MLST groups can be utilized to determine the characteristics of outbreak subtypes. When employed alongside traditional epidemiological methods, MLST characterization can enhance the conclusions of traceback studies after an outbreak.

Cyclospora subtyping has recently become available *(77)*. Isolates from China contained significantly different subpopulations from those in the United States and Peru. The U.S. and Peruvian isolates were genetically similar. This is likely due to the relationship between South American produce imported to the U.S. and foodborne cyclosporiasis *(76)*. Future genotyping will further elucidate the genetic relatedness of *Cyclospora* isolates from around the world.

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CHAPTER 2

DETECTION AND CHARACTERIZATION OF PROTOZOAN PARASITES IN WATER AND BIOFILM SAMPLES FROM AN IRRIGATION WATER SYSTEM[1]

[1] Wakeley, K. and Y. R. Ortega. To be submitted to *The American Journal of Tropical Medicine and Hygiene.*

Abstract

Cyclospora cayetanensis, Giardia duodenalis, and *Cryptosporidium* spp. are protozoan parasites of public health importance. Each parasite has been linked to food and waterborne transmission. Imported fresh produce can carry pathogens to the United States. Irrigation water samples destined to irrigate fresh produce were analyzed for the presence of the protozoan parasites. The objective of this study was to identify links between the parasites and location, seasonality, and climactic factors as well as to determine if water (filtration) or biofilm (toothbrush or swab) sampling tools were more effective parasite collectors. No association was found between climate (average temperature or rainfall) and parasite detection. Location before or after a city did significantly impact the collection the parasites; *Giardia* and *Cyclospora*-like isolates were more commonly found at locations 3 and 6, downstream of small cities. Each parasite was associated with the season of the year. *Giardia* was more associated with Autumn (October-December). *Cyclospora,* and *Cryptosporidium* spp. were more associated with Spring (April-June). Water and biofilm sampling did not collect significantly different amounts of parasites. Comparisons of filter to swab, filter to brush, and brush to swab yielded no difference in parasite collection between sampling tools. RFLP detected 5 *Cyclospora* positives and sequencing detected 4 *Cyclospora* positive samples. RFLP and sequencing analysis of positive *Cyclospora*-like samples showed a mixture of *Cyclospora, Eimeria,* and *Isospora* species.

In conclusion, biofilm sampling can replace water sampling for parasite detection regardless of if swabs or brushes are the sampling tool utilized. Brush sampling is a cheaper and more widely available alternative to swab sampling of biofilms. Sampling for *Cyclospora* and *Cryptosporidium* spp. should occur during Spring when clinical cases of *Cyclospora* and calving season are high particularly for produce farms downstream of cities or animal farms. Further

subtyping or *Giardia* isolates is needed to determine if the isolates obtained are from human or animal origin. Sequencing of the 500bp 18S rRNA gene can more accurately differentiate between *Cyclospora* and related *Eimeria* and *Isospora* species. Utilizing MLST to subtype environmental samples requires further research to locate mini- and microsatellites that will amplify *Cyclospora* DNA in environmental samples.

Introduction

Few protozoan parasites cause gastroenteritis in humans worldwide. *Cyclospora, Giardia,* and *Cryptosporidium* spp. infections can occur as result of the ingestion of contaminated food or water. Namely, imported foods have been implicated in the spread of these parasites to non-endemic locations. The spread of *Cyclospora* through the global food chain can be traced directly to human fecal contamination as its only host are humans. Contaminated raspberries from Guatemala *(9, 27)* and cilantro from Mexico *(1, 19)* were implicated in previous *Cyclospora* outbreaks in the Unites States. A current outbreak (2018) of cyclosporiasis in Minnesota, Iowa, Wisconsin, and Michigan is attributed by epidemiological evidence to Del Monte vegetable trays containing broccoli, dill dip, carrots, and cauliflower *(10)*. *Giardia* and *Cryptosporidium* have other animal hosts that can amplify their presence in the environment. For example, cattle *(3, 17, 49)* and beavers *(13, 16)* are known primary hosts for pathogenic *C. parvum* and *G. duodenalis*, respectively. These hosts can shed their [oo]cysts into the environment through their feces, perpetuating the fecal-oral contamination cycle.

Contamination of irrigation waters is of primary concern in areas that have intensive produce farming, animal farming, and urban developments close to each other increasing the risk of produce contamination through contaminated irrigation waters. Irrigation water testing is necessary at the farm level to understand the pathogens common to the area and to adjust food safety measures accordingly. Sampling regimes for surface irrigation waters has traditionally required filtering large amounts of irrigation water through special filters. U.S. EPA 1623 method *(15)*, the industry standard, utilizes a filter costing $127.00 *(4)*. This method can be cumbersome, time intensive, and cost-prohibitive for small and medium sized farms. Biofilm sampling offers a simpler and cost-efficient method for water sources as biofilms can sequester

the protozoan parasites *(25, 28, 32)*. The tools that will be examined for biofilms sampling are lab grade swabs and firm toothbrushes.

Sequencing and PCR-RFLP *(30)* were employed to characterize positive *Cyclospora*-like isolates. A multilocus sequencing typing (MLST) technique *(24)* was developed to subtype global *Cyclospora* clinical isolates. The use of this technique to subtype environmental *Cyclospora* isolates will be examined.

Methods

Sample Collection

Samples were collected monthly between February 2017 and March 2018 using toothbrushes, swabs, and water filters from 6 different locations in 3 rivers that supply irrigation water to nearby farms. The locations on each river were chosen to assess the microbial quality of the water before and after cities adjacent to their banks. The cities had populations ranging from 1,300 to 2,000 people. None of the cities had adequate wastewater management.

A 2 by 2-inch section of biofilm was removed from river surfaces using the toothbrushes and swabs. Each location was sampled with the swabs and toothbrushes resulting in 24 toothbrush and 24 swab samples per month. Ten gallons of river water from the middle of the rivers was collected. The water samples were taken back to the labs and filtered through Sawyer filters (hollow-fiber, 0.02-0.1 um pore size) over an average of 1.5 hours resulting in 1 filter per sampling site. A total of 54 samples were collected and further processed in the Parasitology Laboratories at the Center for Food Safety.

Sample Preparation

Toothbrushes

The toothbrushes were transferred to individual 50mL centrifuge tubes. The transport bags were rinsed with 3mL of deionized water. The rinse water was added to the 50mL centrifuge tube with the toothbrush and vortexed to remove any debris from the toothbrush bristles. A 0.5mL sample was taken for future virus analysis. The water was then transferred to a 15mL tube. The toothbrush was vortexed 2 more times with 4mL and then 3mL of deionized water. The water was transferred to the 15mL tube after each vortex. The final sample volume was between 10-11mL and stored at 4°C.

Swabs

Each swab was rinsed and vortexed 3 times with deionized water to loosen debris and the rinse water was transferred to a 15mL tube. The first wash used 3mL to clean the swab. A 0.5mL was taken to for future virus analysis. The second and third rinse used 4mL and 3mL, respectively. Approximately 10-11mL of rinse water was collected in the process and stored at 4°C.

Water Filters

The filter was tapped multiple times to release any macroparticles. A syringe filled with 10mL of deionized water was used to rinse the filter and the effluent was caught in a 50mL tube. A 0.5mL sample was taken from the first effluent for further viral analysis. Subsequent washes with deionized water were performed and collected in three 50mL tubes or until the effluent ran clear.

Sample Washing and Concentration

Once all the samples were rinsed and collected in either 15mL or 50mL tubes, each tube was centrifuged at 3000 rpm for 25 mins. The supernatant was discarded and the pellet resuspended in 14 mL of phosphate buffer solution (PBS). The samples were centrifuged again at 3000 rpm for 25 minutes. The pellets were resuspended for a second time in 2.5mL of PBS. Aliquots of 500µL were added to 3 vials designated for DNA extraction, DNA archive, storage with 2.5% potassium dichromate, and 1 mL for ethyl acetate concentration.

DNA Extraction

DNA was extracted following the manufacturer's instructions (FastDNA™ Spin Kit for Soil, Solon, OH) with the following modifications: 200µL of sample and 20µL of 10% milk were added to the Lysing Matrix E tubes; all centrifugation steps occurred at 13,000 rpm for the maximum time recommended; the 15mL tubes were placed in a rack for 10 minutes after inversion to allow for proper settling of the silica binding matrix; DNA was eluted from the spin filter with 65µL of DNase/pyrogen-free water and stored at -20°C until it was used for PCR assays.

PCR Protocols and RFLP Digestion

Cyclospora cayetanensis

Two methods currently being used for *Cyclospora* detection in clinical and environmental samples were used in the present study. Nested PCR amplification was performed based on methods described by Zhou et al. *(53)* and amplified a 501bp fragment of the 18S rRNA gene. A separate 300bp nested PCR-RFLP was performed to differentiate *Cyclospora* species from

closely related apicomplexans like *Eimeria* spp. followed by *Mnl*I enzyme digestion of the 300bp nested PCR product *(30)*. Primer sequences are listed in Table 2.1.

Cryptosporidium spp.

Nested PCR amplification was performed based on methods described by Xiao et al. *(52)* and amplified an 819-825bp fragment of the 18S rRNA gene. Primer sequences are listed in Table 2.1.

Giardia duodenalis

Nested PCR amplified a fragment of the triosephosphate isomerase (TPI) gene approximately 500bp in length *(48)*. Primer sequences are listed in Table 2.1.

Table 2.1. Primer sequences for PCR amplifications of *Cyclospora cayetanensis, Giardia duodenalis,* and *Cryptosporidium* spp.

Gene and Parasite	Gene Position	Primer Name	Primer Sequence - 5' to 3'
18S rRNA			
Cyclospora cayetanensis	519	External-Forward	AAT GTA AAA CCC TTC CAG AGT AAC
	1535	External-Reverse	GCA ATA ATC TAT CCC CAT CAC G
	578	Internal-Forward	AAT TCC AGC TCC AAT AGT GTA T
	1074	Internal-Reverse	CAG GAG AAG CCA AGG TAG GCR TTT
Cryptosporidium spp.	156	External-Forward	TTC TAG AGC TAA TAC ATG CG
	1478	External-Reverse	CCC ATT TCC TTC GAA ACA GGA
	192	Internal-Forward	GGA AGG GTT GTA TTT ATT AGA TAA AG
	1029	Internal-Reverse	AAG GAG TAA GGA ACA ACC TCC A
Triosephosphate Isomerase			
Giardia duodenalis	-5	External-Forward	AAA TIA TGC CTG CTC GTC G
	592	External-Reverse	CAA ACC TTI TCC GCA AAC C
	16	Internal-Forward	CCC TTC ATC GGI GGT AAC TT

	545	Internal-Reverse	GTG GCC ACC ACI CCC GTG CC
PCR-RFLP			
Cyclospora cayetanensis	419	External-Forward	TAC CCA ATG AAA ACA GTT T
	1053	External-Reverse	CAG GAG AAG CCA AGG GAG G
	685	Internal-Forward	CCT TCC GCG CTT CGC TGC GT
	978	Internal-Reverse	CGT CTT CAA ACC CCC TAC TG

Multilocus Sequence Typing (MLST)

A nested PCR targeting five microsatellite loci, Cyc3, Cyc13, Cyc15, Cyc21, Cyc22, was performed for confirmed *Cyclospora cayetanensis* clinical isolates from Nepal, Mexico, and Peru following the protocol described by Guo et al. *(24)*. The amplified loci were sequenced to determine genetic diversity at the targeted loci and compared to global *Cyclospora* sequences in GenBank. Primer sequences can be seen in Table 2.2.

Table 2.2. Primer sequences for Multilocus Sequence Typing (MLST) of *Cyclospora cayetanensis*

Gene and Parasite	Primer Name	Primer Sequence - 5' to 3'
Multilocus Sequence Typing		
CYC3	External-Forward	GAA GAT GAA GCG TTG GTA CG
	External-Reverse	TAC CGC TGC TGG AGT GCA T
	Internal-Forward	TTG TGC ATG GCA CCC AAT GC
	Internal-Reverse	CCA GAC AGT AGT TCG TGT CTT
CYC13	External-Forward	TTG GAG CAG GAC GAG TTT CG
	External-Reverse	ATG GAA GCG GCT ATG AAA TTG G
	Internal-Forward	CCT CGG AGT CCT CTG AGT G
	Internal-Reverse	AGC CGT CGC AGT GTG TAG CA
CYC15	External-Forward	AGT AGC TAC GTG CCA AGA CGA
	External-Reverse	TCG TTC TAT CTG ACC ATA GTA GTG
	Internal-Forward	CGC TGT GCA AGA GGC GAT CTA
	Internal-Reverse	AAG CAC TGC AGG GTC CGT AA
CYC21	External-Forward	TAG TGG CGA CTG CGA CAT G
	External-Reverse	GCA CCT TGC TGA TGA GGC A
	Internal-Forward	CTA AGG CTG TCT TGA GCG G

	Internal-Reverse	CGC CCA CAT GCT TCG TAT AC
CYC22	External-Forward External-Reverse Internal-Forward Internal-Reverse	CAC TAT GCC GTG TGA CAC GT GTA GAT TTG CAA GAA CTC ATG CTA ATA GTA TTC AGG CGC AAA CTA AG GAG GCT TTC CAA AGG TCT AGT T

Sequencing

The final PCR products for *Cyclospora* spp. 500bp PCR amplification and MLST microsatellites were purified using the QIAquick PCR Purification Kit (Maryland, USA 20874) according to the manufacturer's instructions with one modification: water was used to elute DNA from the spin filter. The concentration of the purified DNA was then measured using a NanoDrop 1000 (Wilmington, Delaware 19810). 20 ul of the DNA (20ng/ul) was sent for sequencing to Macrogen USA Corp. (Rockville, MD 20850) using the nested forward and reverse primers. Sequences were assembled using ChromasPro software and aligned with each other along with reference sequences obtained from GenBank using ClustalX. Unique sequences were deposited into GenBank.

Statistical Analysis

Each sample was run in triplicate for PCR. A sample was considered positive if one triplicate was positive. The results were analyzed using Microsoft Excel and JMP Pro. Temperature and rainfall data were retrieved from Servicio Meteorológico Nacional. The wet and dry season was defined as May to October based on the average rainfall per month. Chi-square and Fischer's Exact Test were utilized to determine the relationship between parasite collection and the sampling tool, sampling medium, location, and season.

Results

Positive *Cyclospora*-like*, Cryptosporidium* spp., and *Giardia* samples were collected in 38.1%, 23.7%, and 24.7% of samples, respectively. There was no significant difference between water and biofilm sampling for collecting any of the parasites. There was no significant difference between water and swab, water and brush, or swab and brush sampling methods for any parasite collection or collection of the parasite individually. Detection of *Cyclospora*-like, *Cryptosporidium* spp., and *Giardia* positive samples were not significantly different between water and biofilms. Figure 2.1. shows the relationship between parasite collection and sampling tool.

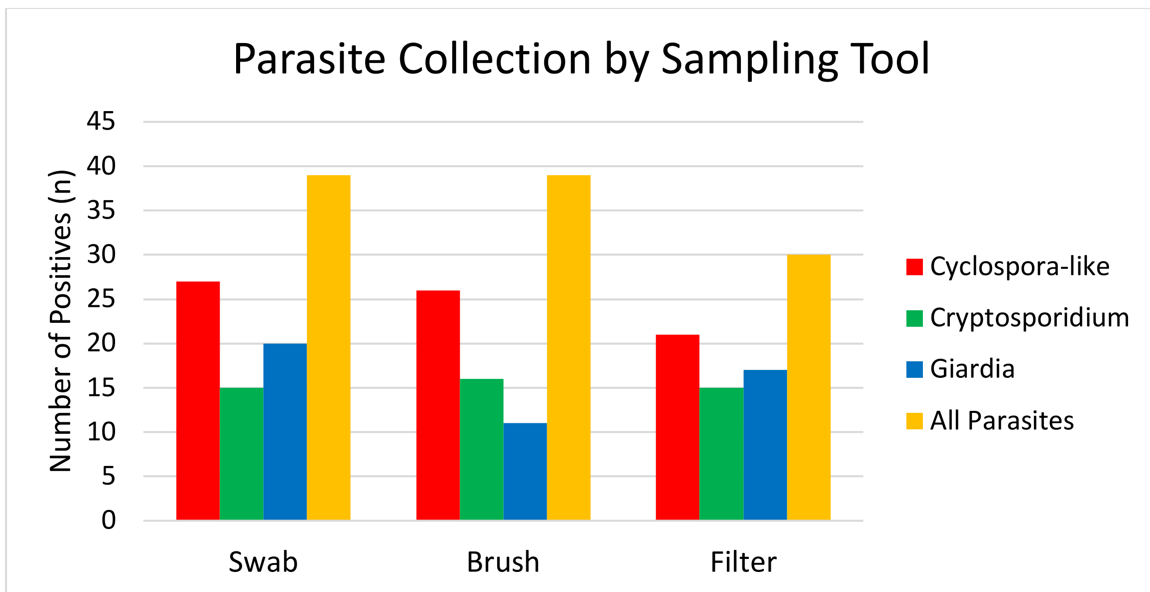

Figure 2.1. Distribution of *Cyclospora*-like*, Cryptosporidium* spp., and *Giardia* frequency by each sampling tool

Location was significantly associated (n= 108; p<0.0001) with parasite detection using all sampling methods. Locations 1 and 2 were along River A, locations 4 and 3 on River B, and

locations 5 and 6 were on River C (Figure 2.2). Significantly more parasites were detected at locations 3 (n=24; p=0.0211) and 6 (n= 29; p<0.0001), both located after a city, than their upstream counterparts, locations 4 (n=14) and 5 (n=12), respectively. There was no significant difference between locations 1 (before; n=12) and 2 (after; n= 17). *Cyclospora*-like and *Giardia* positive samples were more likely collected at downstream sites 3 (n=17, p=0.0017; n=14, p=0.0272) and 6 (n=22; p=0.0002; n=17, p=0.0006) than upstream sites 4 (n= 5; n=5) and 5 (n=7; n=4). There was no significant difference between the upstream locations and downstream locations for *Cryptosporidium* spp. collection.

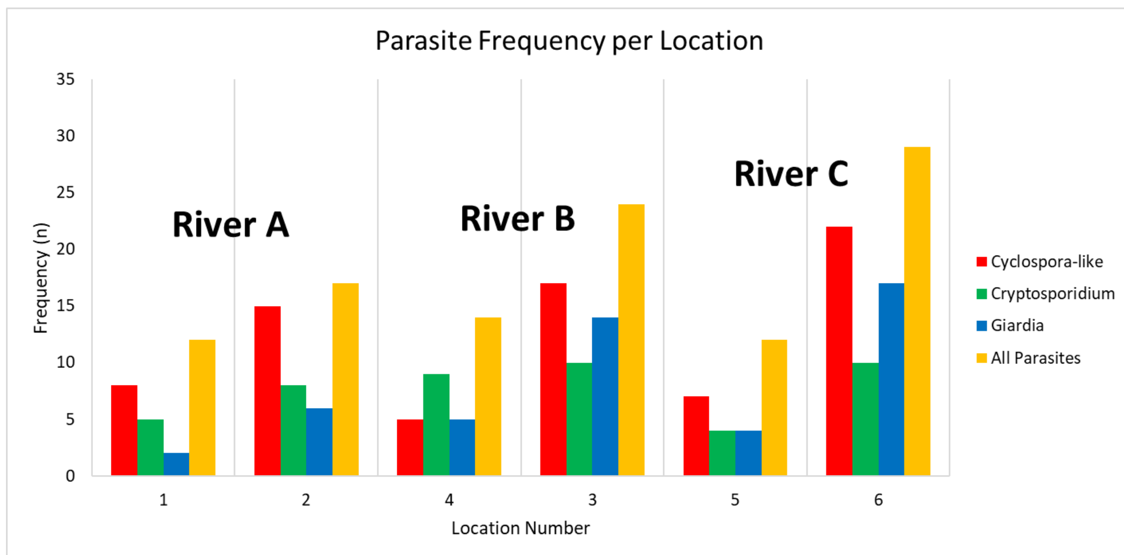

Figure 2.2. Location and corresponding parasite collection frequencies

Parasite collection for all the parasites was not associated with the wet and dry seasons, average rainfall per month, or the monthly temperature of the region.

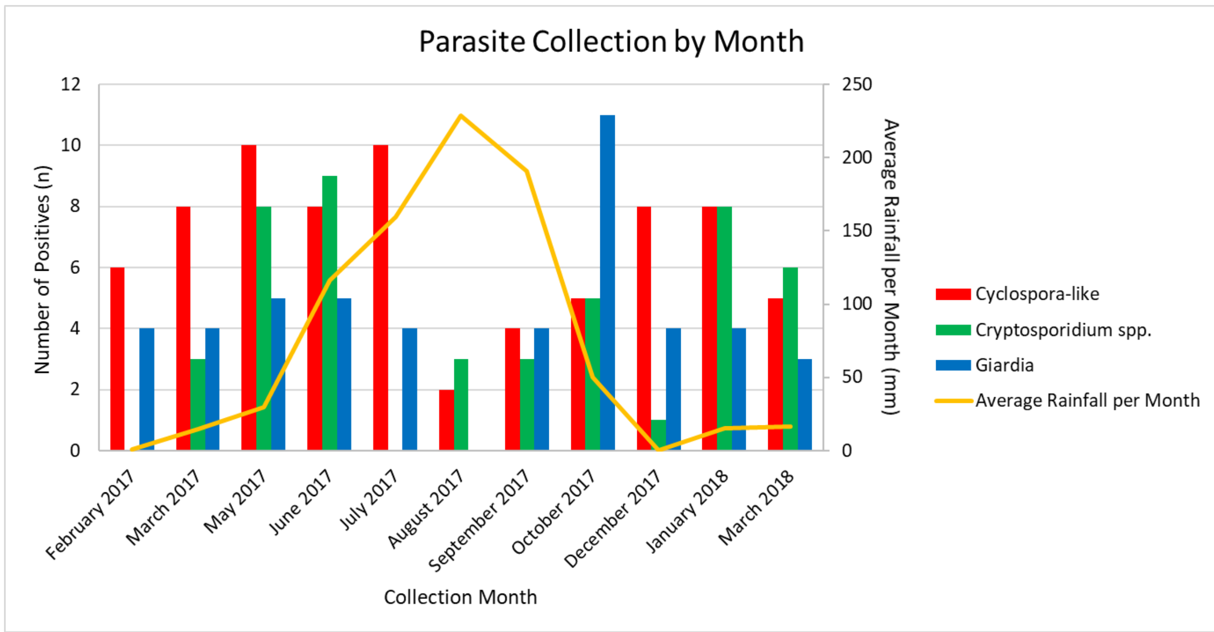

Figure 2.3. Plot of the *Cyclospora*-like*, Cryptosporidium* spp., and *Giardia* counts per month and the average rainfall per month

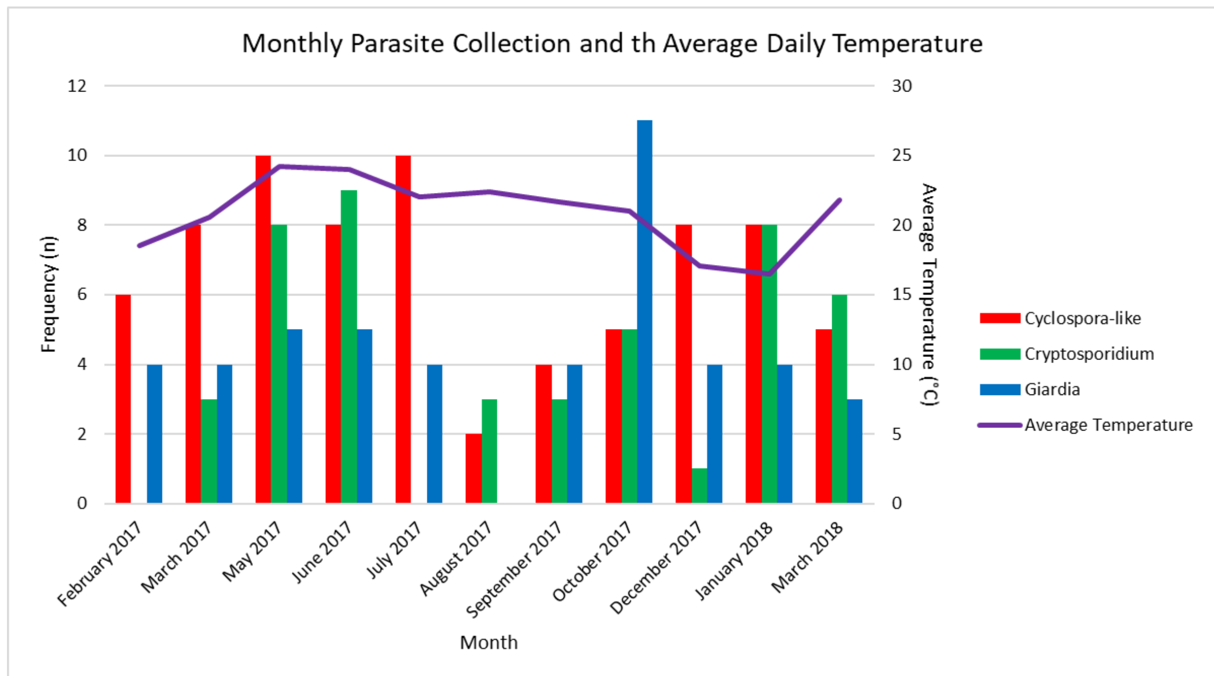

Figure 2.4. Graph of the average monthly temperature and parasite collection

Parasite collection counts can be seen in Figure 2.2. *Cyclospora*-like collection increased between February 2017 and July 2017 with a sharp decrease in August 2017. Collection counts steadily increased through January 2018 and slightly decreased in March 2018. *Cryptosporidium* spp. collection peaked in June 2017. Zero samples were positive for *Cryptosporidium* spp. in February 2017 and July 2017. *Cryptosporidium* spp. collection steadily increased from August 2017 to October 2017, decreased in December 2017 and March 2018, and January 2018. *Cryptosporidium* spp. and *Giardia* detection were significantly associated with all the seasons. *Cryptosporidium* spp. was detected more in the Spring (April-June) (n=17/32) than in the Winter (January-March) (n=17/72; p=0.0035) or Summer (July-September) (n=6/54; p<0.0001). *Giardia* was detected primarily in the Autumn (October-December) (n=15/36) than the Summer (n=8/54; p=0.0047) and the Winter (n=15/72; p=0.0213). *Cyclospora*-like detection occurred more in the Spring (n=18/32) than the Summer (n=16/54; p=0.0136). There was no difference between Winter/Spring detection for *Cyclospora*-like and *Giardia* detection, Summer/Autumn or Winter/Autumn detection for *Cyclospora*-like and *Cryptosporidium* spp., or Spring/Summer detection for *Giardia*.

Of the 103 *Cyclospora*-like positive samples obtained by PCR, 52 samples were identified by PCR-RFLP; the other 51 samples could not be amplified using the PCR-RFLP protocol (Table 2.3). Five samples were identified as *Cyclospora* spp. by PCR-RFLP. A developed PCR-RFLP gel can be seen in Figure 2.5. All 103 *Cyclospora*-like samples were sent for sequencing. Nineteen of the samples were not successfully sequenced. The remaining 84 samples were a mixture of *Cyclospora, Isospora,* and *Eimeria* species as seen in Table 2.4. Three of the sequencing samples were definitively *Cyclospora cayetanensis* compared to the GenBank

sequences. Twenty-six of the PCR-RFLP sample results matched with the sequencing results and twenty-six did not.

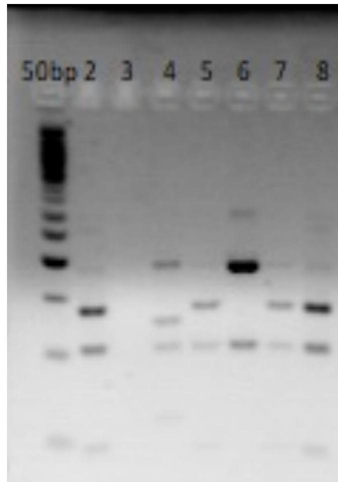

Figure 2.5. Example of a developed PCR-RFLP gel: Lane (1) 50 bp Marker, (2) *C. cayetanensis* positive, (3) sample 366 did not develop, (4) sample 370 *E. tenella*, (5) sample 374 *C. cayetanensis*, (6) sample 396 *Eimeria* spp., (7) sample 425 *C. cayetanensis*, (8) *C. cayetanensis* positive

Table 2.3. PCR-RFLP results for *Cyclospora, Isospora,* and *Eimeria* species detected in the *Cyclospora*-like positive samples by sampling tool

RFLP Results	Total	Brush	Swab	Filter
Total Amplified	52	21	21	10
E. tenella	11	7	1	3
Eimeria spp.*	33	11	17	5
Cyclospora spp.	5	1	2	2
Not Amplified	51	18	22	11

Table 2.4. Sequencing results for *Cyclospora, Isospora,* and *Eimeria* species detected in the *Cyclospora*-like positive samples by sampling tool

Sequencing	Total	Brush	Swab	Filter
Total Sequenced	79	27	35	17
Eimeria spp.*	61	19	30	12
Isospora spp.	11	7	3	1
Cyclospora cayetanensis	3	0	2	1
Eimeria spp. + *Cyclospora* spp.	1	0	0	1
Eimeria spp. + *Isospora* spp.	2	1	0	1
Isospora spp. + *Cyclospora* spp.	1	0	0	1
Not Sequenced	24	12	8	4

**Eimeria* spp. detected include *Eimeria papillate, Eimeria acervulina, Eimeria scholtysecki, E. cahirinensis, Eimeria kanyana, Eimeria telekii, Eimeria lancastarensis, E. bukidonensis, E. nieschulzi, E. apionodes, E. ferrisi, E. albigulae, E. langebarteli, E. leucopi, Eimeria apodemus, Eimeria arizonensis, E. scholtysecki, Eimeria banffensis, Eimeria maxima, Eimeria hirci, E. crandallis, Eimeria necatrix, Eimeria mitis, E. mitis, Eimeria christenseni, E. ahsata, E. albiqulae,* and *E. bukidonensis.*

The positive *Cyclospora cayetanensis* environmental samples were subtyped using MLST. None of the *Cyclospora* environmental isolates could be subtyped using the MLST tool described (Figure 2.6).

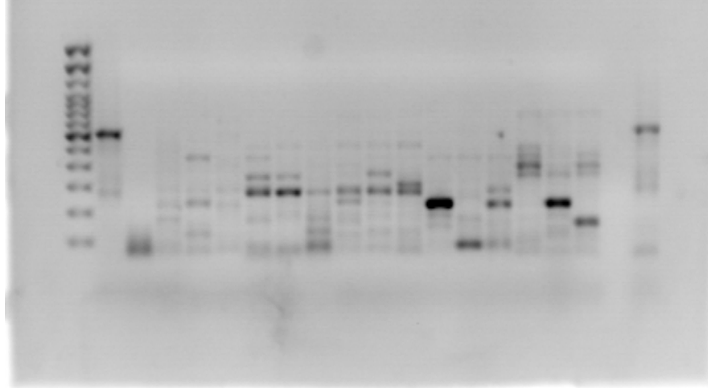

Figure 2.6 Amplification of environmental confirmed *Cyclospora* isolates using MLST primer CYC3. The 2nd and last lanes are the *Cyclospora* positive controls.

Discussion

Irrigation water quality varies through the seasons and is highly dependent upon surrounding land use and municipal sanitation regimes. *Giardia, Cryptosporidium* spp., and *Cyclospora* are the most commonly detected protozoan parasites in water samples from Latin America and are of significant public health importance to the region *(44)*. *Cyclospora* in the Americas is endemic to Mexico, Guatemala, Haiti, Dominican Republic, Venezuela, Colombia, Peru; previous international outbreaks have been associated with travel and food imported from endemic areas *(1, 19, 36, 50)*. Foodborne cyclosporiasis in the U.S. is associated with eating imported fresh produce *(8, 9, 19)*. Previous studies have established irrigation water as a source of enteric pathogens on produce. Gemmel et al. *(21)* postulated that fecal coliforms from the Baynespruit River in South Africa were contaminating fresh produce and leading to the high concentrations of fecal coliforms found on the produce (1.6 x 10^5 CFU). Furthermore, the district of Lublin, Poland with high levels of cattle homesteads were seen to have higher contamination rates of *Cryptosporidium* spp. on their produce suggesting that irrigation water was the primary transmission vehicle *(45)*. Each farm in the region of study utilized various irrigation methods

(i.e. drip, subsurface, and trench) and irrigation water treatments (i.e. chlorine, UV, filter) increasing or decreasing the risk of contamination to their produce.

Prevalence

Cyclospora-like organisms (38.1%) were collected more than the other parasites. *Cyclospora cayetanensis* was detected in 2.58% of the samples examined in the present study as confirmed by sequencing of the 18S rRNA gene. Similar studies detected *Cyclospora* in other water sources. *Cyclospora* spp. was detected in 6.2% of well water and 21.3% of treated water samples taken from municipal treatment plants in Italy *(22)*. Sampling of influent from a drinking water treatment plant in Malaysia detected *Cyclospora* in 8.33% (2/24) and 17% (2/12) of recreational water samples *(6)*. *Cryptosporidium* (18%) rates were in line with rates detected in a Mexican agricultural canal *(51)*. However, the *Giardia* rates (64%) were nearly three times higher than was detected (24.7%) in the same study. *Giardia* and C*ryptosporidium* spp. rates in other Latin and South American countries varied from the ones reported here. Samples of raw water entering a wastewater treatment plant in Londrina, Brazil detected lower rates of *Cryptosporidium* spp. in 2 (8.33%) and *Giardia* in 2 (8.33%) of the samples *(2)*. Another Brazilian study performed on the Atibaia River detected significantly greater numbers of *Cryptosporidium* spp. (62.5%) and *Giardia* (62.5%) *(38)*. *Cryptosporidium* spp. was recovered from the raw water samples in Puerto Rico at a rate of 33% (12/36) *(43)*.
Giardia and *Cryptosporidium* spp. are common to surface waters globally. Raw water samples taken from the Blankaart catchment in Belgium detected *Giardia* in 92% and *Cryptosporidium* spp. in 96% of the samples. Human waste was considered a source of contamination as one of the sampling sites was downstream of a wastewater treatment facility *(14)*. An Iranian study

detected *Giardia* in 13 (65%) and *Cryptosporidium* spp. in 6 (30%) of samples collected along the Sefidrood River *(35)*. *Giardia* and *Cryptosporidium* spp. were detected in 52% and 25%, respectively, of samples taken from the Kuang River in Northern Thailand *(11)*. Likely the difference of prevalence rates of the parasites in each country is due to differences in surrounding land usage, availability of wastewater treatment measures, and the prevalence of the parasites in human and animal hosts in the area.

Seasonality

Interestingly, there was no significant difference between parasite detection in the wet and dry seasons or an association with average rainfall per month. of the study area. Parasite collection seemed to decrease with increasing rains as seen in Figure 2.2. This is likely due to the monthly rains diluting the existing concentrations of the parasites in the river systems resulting in lower sampling yields.

Other tropical and subtropical countries have shown a clear association between *Cyclospora* infections and the wet and dry seasons. Thailand demonstrates a connection between *Cyclospora* collection and the wet and dry seasons. Nearly double the oocysts were collected in the wet (40%) than the dry (21%) season *(11)*. Guatemalan stool samples revealed an association between higher rainfalls and *Cryptosporidium* spp. prevalence in clinical stool samples *(5)*. A Honduran study found that 83.3% of the *Cyclospora* infections from 2002-2011 occurred during the rainy season *(31)*. Further research may illuminate how environmental factors like water chemistry and climate affect *Cyclospora* persistence and recovery in water systems.

Each parasite was associated with a season of the year. *Cyclospora*-like detection occurred primarily in the Spring (April-June) and coincides with the seasonal increase of

Cyclospora clinical cases in North America. Children in Morelia, Mexico were observed to have *Cyclospora* during June-August *(39)*. Other Latin American countries show an increase of *Cyclospora* cases in the warmer months. Both Guatemalan *(5)* and Honduran *(31) Cyclospora* cases increased between May and August.

Giardia detection was associated more with Autumn (October-December) than the other seasons. A previous study demonstrated an annual increase of clinical giardiasis between May and September in Mexico *(26)*. Clinical samples taken from Oceania, Europe, Canada, U.S., and U.K. show an increase of *Giardia* cases in the summer *(33)*. Prevalence of *Giardia* in Latin American water ways increased during the spring and summer *(44)*. An Egyptian study found increases in giardiasis cases in mid-summer and late-winter *(29)*.

Cryptosporidium spp. significantly increased in the Spring over the other seasons. U.S. and Canadian peaks in cryptosporidiosis cases occurred primarily in the late summer with a small spike in spring for Canada *(33)*. An increase in water recreation that occurs in the summer is considered a contributing factor to the higher number of cryptosporidiosis cases seen during these months *(40)*. In addition to growing produce, the study area is one of the top cattle producing states in Mexico *(41)*. Calf rearing in the spring could contribute to the increase of *Cryptosporidium* spp. in nearby irrigation waters. An increase of cryptosporidiosis cases during calving season was also observed in New Zealand *(20)*. Collection of the parasites was not correlated with the average monthly temperature. The little fluctuation in the temperature throughout the year, as seen in Figure 2.3, is the likely cause of this phenomenon.

Biofilm and Water Sampling

Water and biofilm sampling were not statistically different from each other. Biofilm sampling can serve as an alternative to water sampling methods for farmers and public health professionals

seeking to sample river systems. Brush or swab sampling of biofilms is convenient to the sampler, compact, and requires little pre- or post- sampling preparation. The ease of biofilm sampling over water sampling decreases the effort needed for farmers to investigate the types of pathogenic protozoa in their irrigation waters. The increasing risk that protozoan parasites play to the globalized food market make determining simpler parasite sampling methods in irrigation waters imperative to global health. This is particularly important where surface waters receive no additional interventions before being used in the field.

Biofilms containing parasites present a public health concern. *Giardia duodenalis* and *Cryptosporidium parvum* [oo]cysts can attach to established biofilms in drinking water distribution systems within an hour of their introduction and remain viable up to 34 days *(25)*. Changing waterflow conditions can have varying effects on *Giardia* and *Cryptosporidium* spp. detachment from lab-created biofilms. Laminar flow conditions showed little change in *Giardia* and *Cryptosporidium* spp. concentrations *(25, 46)*. However, changing from laminar flow to turbulent flow showed an increase of parasite concentration in the water phase of drinking and wastewater system biofilms *(25)*. This suggests that large changes in flow conditions around biofilms can result in detachment and a possible contamination event.

Furthermore, protozoan parasites may enter a river system attached to fecal or soil particles or quickly associate with river particles once in the system *(12)*. Once associated with these particles, the parasites are seen to settle more quickly than planktonic parasites *(18)* depending on hydrological conditions. Physicochemical changes within an aquatic environment can affect the formation of these particles and their interactions with other riverine substrates like biofilms *(12)*. Further investigations into biofilm sampling should consider the attachment characteristics of the protozoan parasites to other substrates within river systems (i.e. particles,

soils, other microorganisms) and how these characteristics can change according to the physicochemical properties of the environment.

Location

The downstream sampling sites 3 and 6 had significantly higher *Cyclospora*-like collection than their upstream counterparts. Humans are the only hosts for *Cyclospora.* Its association with locations downstream of small cities suggests human wastes from the cities is contaminating the waters. Poor sanitation of human wastes is likely the cause of *Cyclospora* contamination in the rivers. Contamination of river systems with city wastes is common. Eutrophication and contamination of irrigation water systems with human wastes around Mexico City have resulted in low irrigation water quality for farms operating downstream of the city *(37)*. Ribas et al. *(42)* found an increase in enteric pathogens downstream of the sample village demonstrating the impact the village was having on water quality.

The presence of *Giardia* in the rivers was associated with downstream locations 3 and 6. *Giardia* is the most common enteric parasite in humans globally *(7)*. This may be the result of a recent giardiasis outbreak in the city upriver or due to another confounding variable. Subtyping og the *Giardia* isolates collected would clarify the origin of the assemblages as possibly human or animal. This information would further determine if human fecal waste was the cause of the higher *Giardia* rates at locations 3 and 6. *Cryptosporidium* spp. detection was not associated with upstream or downstream locations likely due to its cosmopolitan host range and ubiquity in nature.

Restriction Fragment Length Polymorphism

PCR-RFLP is a clinical technique used to differentiate *Cyclospora cayetanensis* from closely related *Eimeria* spp. Only 50% of the *Cyclospora*-like positive samples could be amplified using the PCR-RFLP technique. PCR-RFLP amplification uses primers that amplify generic *Cyclospora* spp. and are not specific to *C. cayetanensis (47).* Sequence data was sufficient to type *Cyclospora* spp., *Eimeria* spp., and *Isospora* spp. that cross-reacted with the nPCR primers. The failings of PCR-RFLP may be reflective of the enzyme used to fragment the amplified DNA, *Mnl*I. Using the *Kpn*I enzyme, Li et al. *(34)* found that PCR-RFLP could differentiate between cattle associated *Eimeria* spp. and *Cyclospora*-like oocysts with gel differentiation. Improvements to the standard PCR-RFLP method could include more specific primers and an enzyme that fragments the amplified DNA in a way that can differentiate *Eimeria* and *Cyclospora* species.

Sequencing of the 500bp 18S rRNA gene returned results for about 77% of the samples. Our sequencing data confirms that RFLP is not a sensitive method for *C. cayetanensis* confirmation and does not accurately genotype the species found in environmental samples. The five samples genotyped as *C. cayetanensis* by RFLP were revealed to be *Eimeria maxima* (n=1)*, Isospora spp.* (n=3)*,* and *Eimeria mitis* (n=1) by sequencing analysis. Furthermore, three samples that did not amplify for PCR-RFLP genotyped as *C. cayetanensis* by sequencing.

The sequencing results also compared larger gene fragments of environmental samples to global *Cyclospora* sequencing data in GenBank. The information uploaded to the database can help future researchers recognize species that cross react with the *Cyclospora* 500bp PCR primers. This data could be used at a future date to make more specific primers for the identification of *Cyclospora* in environmental samples. Continued research into PCR-RFLP

components is necessary to increase the sensitivity of the method. The advantages of PCR-RFLP is the method’s relative ease of use, quick results, and similar skill level to other PCR identification methods. It can serve as a cheaper, preliminary diagnostic tool for individuals who do not have access to cost-effective, quick, or reliable access to sequencing facilities.

Multilocus Sequence Typing (MLST)

MLST is a tool used to determine the relatedness of strains from the same species. Five gene loci were identified as useful for subtyping clinical *Cyclospora cayetanensis* samples *(23, 24)*. These loci did not amplify *Cyclospora* in environmental samples. This is likely due to low concentrations of *Cyclospora* DNA in the environmental samples. Further research is necessary to create a subtyping tool that is sensitive to smaller concentrations of *Cyclospora* DNA.

Conclusions

Produce farms downstream of urban developments and animal farming are vulnerable to pathogen contamination and should consider creating a sampling plan for enteric pathogens. Water and biofilm sampling equally collect parasites from an irrigation system destined to irrigate fresh crops. Biofilm sampling is a simpler, more maneuverable, and cost-effective method for irrigation water sampling of parasites. The two biofilm sampling methods examined, brushes and swabs, had comparable results. The brushes represent an available and cheaper alternative to the lab-grade swabs. *Cyclospora*-like organisms and *Giardia* sampling should occur April-August and May-September, respectively, when clinical cases are at their highest, particularly for farms downstream of populated areas. *Cryptosporidium* spp. sampling should begin in the early spring and end in October when calves are weaned and human cases decrease.

Preventative methods are necessary to reduce contamination of the edible portion of produce. Surface and sub-surface irrigation methods, proper handwashing and bathroom facilities, an emphasis on employee hygiene, outer covering like gloves and face masks, and pest control are equally necessary interventions to prevent produce contamination on farms.

Sequencing of suspected *Cyclospora* isolates is shown to be more accurate than PCR-RFLP identification and could replace the technique. Current PCR-RFLP techniques are not specific to *C. cayetanensis.* Despite this, PCR-RFLP represents a cost-effective, more accessible alternative to sequencing that areas without access to reliable sequencing facilities could employ to differentiate *Cyclospora*-isolates from other species. Further research needs to be done to improve the specificity of the current PCR-RFLP methods available for *Cyclospora.* Utilizing MLST to subtype environmental samples requires further research to locate mini- and microsatellites that will amplify *Cyclospora* DNA in environmental samples.

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