DETECTION AND GENOTYPING OF *CRYPTOSPORIDIUM SPP.* IN BIOFILMS AND CATTLE FECES FROM THE BLACK RIVER WATERSHED, JAMAICA

by

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Detection and Genotyping of *Cryptosporidium spp.* in Biofilms and Cattle Feces Collected from the Black River Watershed, Jamaica

Doctor of Philosophy, Amanda Morris, Environmental Applied Science and Management, Ryerson University, 2019

Abstract

Cryptosporidium is a protozoan parasite that causes the gastrointestinal disease cryptosporidiosis. The disease is endemic in most tropical countries including Jamaica; yet underreported from environmental sources. This is of concern because the primary source of anthropocentric cryptosporidiosis is surface water contaminated by human effluent and animal waste in runoff. This dissertation therefore, focuses on three main areas of research: 1) optimization and assessment of effective methods for detecting *Cryptosporidium* from environmental samples; 2) application of methods to analyze biofilm and cattle feces collected from the Black River watershed, located in the rural parish of St. Elizabeth, Jamaica; 3) molecular characterization of PCR-positive detections to identify *Cryptosporidium* species and genotypes, thereby provide inference to waterborne transmission, mitigation, and zoonotic potential within the region. First, foundational work of this dissertation focused on the development of *in situ* biofilm sampling for *Cryptosporidium* detection. Application was then performed in the Black River network for initial screening of oocysts from biofilms collected from 5 sites, and 119 cattle fecal specimens collected from 10 farms. Multiple techniques were employed to confirm the absence or presence of *Cryptosporidium*, including Immunofluorescence Assay (IFA) and Modified Acid-Fast (MAF) microscopy, Enzyme-Linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR). Results show that oocysts were widely dispersed in biofilms and cattle specimens. Highest prevalence was observed amoung dairy cattle compared to beef cattle, presumably due to confined space. Results

also highlight inconsistencies between detection methods, confirming that from environmental sources—where inhibitors are abundant and oocysts concentrations are naturally low—a single technique may be ineffective for understanding transmission dynamics. The second part of this dissertation focused on molecular characterization. Fourteen PCR-positive biofilm and cattle samples were subjected to gene sequencing and phylogeny. A low species diversity consisting of *C. parvum* and *C. hominis* were identified; the two most common species involved in anthropocentric infections. A specific 18S rRNA isolate of *C. parvum* was found in both biofilm and cattle samples (with 99% identity), indicating that a geographically distinct, clonal genotype of *C. parvum* potentially exists within the region. Further subtyping analysis of the gp60 locus identified one *C. hominis* subtype (IbA9G2), formally identified in human populations worldwide. Moreover, the IbA9G2 subtype was recently linked to calf infections in France, as well as a waterborne outbreak in Germany. In conclusion, this dissertation is the first to detect and characterize *Cryptosporidium* species and genotypes from surface water biofilms and cattle feces in Jamaica, providing informative data pertaining to public health and animal agriculture. Moreover, this research advocates the importance of utilizing multiple detection methods and sources for effective screening of *Cryptosporidium* throughout the environment. Whilst meaningful interpretations of *Cryptosporidium* population structures are developed, useful databases can form through analyzing a well-planned set of environmental samples.

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Dedication

To my family, especially my parents Faye and Delvon Morris for your love, inspiration and support. Your actions, words of empowerment and wisdom have taught me to know no boundaries, aim high and believe in myself. Special thanks to my amazing fiancé Michael Cole for your love, support and encouragement throughout this journey.

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List of Abbreviations

AIDS	acquired immune deficiency syndrome
BLAST	basic local alignment search tool
BSA	Bovine Serum Albumin
BS-C-1	monkey kidney epithelial cells
BFTE	bovine fallopian tube epithelial cells
Caco-2	human epithelial colorectal adenocarcinoma cells
CC-PCR	cell culture-Polymerase Chain Reaction
CDC	Centers for Disease Control and Prevention
CFSPH	Center for Food Security & Public Health
DAPI	4',6-diamidino-2-phenylindole
DEM	Digital Elevation Model
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DOC	Dissolved Organic Carbon
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EPS	extracellular polymeric substances
FISH	Fluorescent *in situ* Hybridization
FMS	Faculty of Medical Sciences
GEMS	Global Enteric Multicenter Study

GIS	Geographic Information System
gp60	60-kDa glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	human immunodeficiency virus
HCT-8	human colon epithelial cells
hsp70	Heat shock protein 70
HWTP	Howard Avenue Water Treatment Plant
JPAT	Jamaican Protected Areas Trust Limited
ICR	Information Collection Rule
IOM	Institute of Medicine
IFA	Immunofluorescence Assay
IMS	Immunomagnetic Separation
ICZN	International Code of Zoological Nomenclature
KCF	Kinyoun's Carbol Fuchsin
MAF	modified acid-fast
Mal-ED	Malnutrition and the Consequences for Child Health and Development
MDCK	Madin-Darby Canine Kidney
MPC	Magnetic Particle Concentrator
mRNA	messenger ribosomal ribonucleic acid
MWW	Milwaukee Water Works
NCBI	National Center Biotechnology Information
NEPA	National Environmental and Planning Agency
NIH	National Institute of Health
NOM	natural organic matter

NTU	nephelometric turbidity units
PCR	Polymerase Chain Reaction
PWD	Philadelphia Water Department
RADA	Rural Agricultural Development Authority
REF	Relocatable-grid reference
RT-PCR	reverse transcription-Polymerase Chain Reaction
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviation
S.D.	standard deviation
sp.	species singular
spp.	species pluralis
SSU	small-subunit
TCAG	The Centre for Applied Genomics
TSB	tryptic soy broth
USDA	United States Department of Agriculture
UHWI	University Hospital of the West Indies
UK	United Kingston
US	United States
UV	ultra-violet
UWI	University of the West Indies
WHO	World Health Organization

Chapter 1: Literature Review

1.1. *Cryptosporidium* and Cryptosporidiosis

The genus *Cryptosporidium* is a protozoan parasite that causes the gastrointestinal disease cryptosporidiosis in humans and many other vertebrate species. The term "pervasive" most accurately describes *Cryptosporidium* because the parasite is globally widespread and persists under various climate conditions. In addition, *Cryptosporidium* has been detected in at least 260 vertebrate species from all classes including mammals, reptiles, birds, fishes and amphibians (Fayer & Xiao, 2008). The manifestation of cryptosporidiosis typically occurs when a suitable host ingests *Cryptosporidium* oocysts (spore-like eggs) from fecal-contaminated water, food or other sources. On rare occasions, *Cryptosporidium spp.* have been found in respiratory infections due to the inhalation of emitted aerosols (Spickler, 2013).

Upon ingestion by a suitable host, the most common symptom of cryptosporidiosis is watery diarrhea. Other symptoms include: stomach cramps, dehydration, nausea, vomiting, fever, weight loss, muscle aches and poor appetite (Centers for Disease Control and Prevention [CDC], 2016; Spickler, 2013). The duration and severity of symptoms depend largely on the age and immune status of the individual. In humans, symptoms transpire 2 to 10 days after infection, and can last between 1 and 2 weeks (CDC, 2016a). Similarly, healthy animals experience symptoms a few days after infection, and exhibit sporadic recovery between 1 and 2 weeks (Fayer & Xiao, 2008; O'Donoghue, 1995). Also, some humans and animals carry the disease without showing signs of illness (Cacciò & Widmer, 2013; CDC, 2016; CFSPH, 2014; O'Donoghue, 1995; Spickler, 2013).

In most cases, cryptosporidiosis is characterized as an acute, self-limiting infection. In general,

healthy individuals infected by the disease recover without medical treatment. However, in immunocompromised individuals, cryptosporidiosis can be chronic and life threatening, especially in HIV/AIDS, cancer and transplant patients. For treatment of HIV/AIDS patients, anti-retroviral therapy is usually administrated. The procedure uses multiple drugs to improve the immune status of individuals thereby inhibits *Cryptosporidium*. However, therapeutic treatment is sometimes ineffective and severe symptoms often worsen or lead to death (CDC, 2016a; Fayer & Xiao, 2008). The elderly, young, malnourished and pregnant are also vulnerable to the disease (Cacciò & Widmer, 2013; Putignani & Menichella, 2010). To assist in recovery, fluid rehydration, electrolyte correction and antibiotics such as nitazoxanide are generally prescribed (CDC, 2016a; Fayer & Xiao, 2008).

In animals, the success of antiprotozoal therapy is reportedly inconsistent. Available antiprotozoal medications have either suppressed oocysts shedding or resulted in poor clinical improvements. Also, natural remedies have shown to antidotally reduce the severity of symptoms in livestock; though the effectiveness of this form of treatment is supported by limited scientific research (Shahiduzzaman & Daugschies, 2012).

1.2. General Biology

1.2.1. Taxonomy of the Genus Cryptosporidium

Cryptosporidium is a genus within the Apicomplexa phylum—a group of parasitic eukaryotes that have an apical complex, which is a subcellular structure designed for host invasion and attachment (Gubbels & Duraisingh, 2012; Okamoto & Keeling, 2014). Within the Apicomplexa phylum, *Cryptosporidium* was traditionally classified as a coccidian because of its life cycle phases including sexual reproduction by gamogony and asexual reproduction by merogony and

sporogony (section 1.2.3.). However, ultrastructural studies and molecular evidence now demonstrate that *Cryptosporidium* is not a coccidian, but a gregarine. Figure 1.1. illustrates the taxonomic discrepancy of *Cryptosporidium* classification under the Apicomplexa phylum.

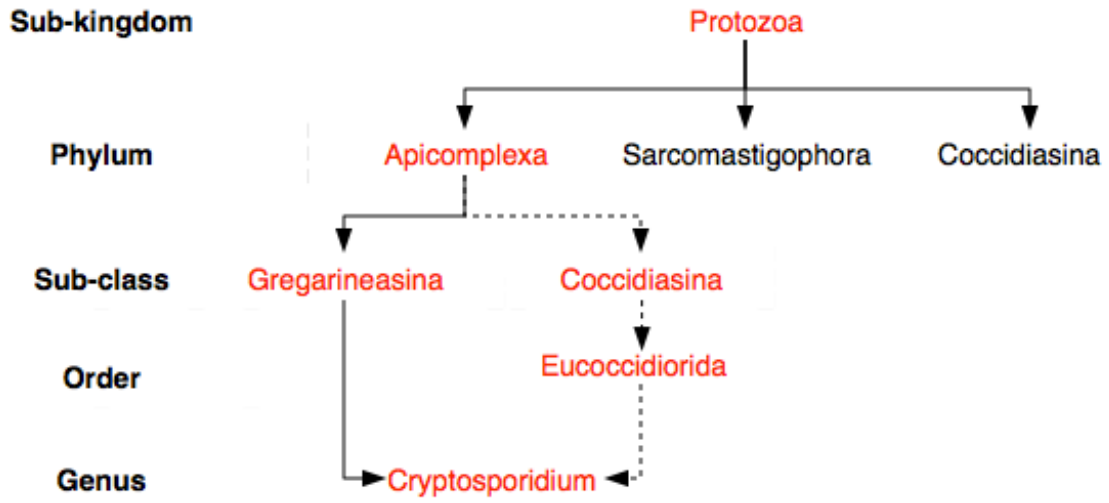

Figure 1.1.: Classification of *Cryptosporidium* in the Apicomplexa phylum. Includes gregarineasina and coccidiasina sub-classes. Broken arrows indicate traditional classification of *Cryptosporidium*. Solid arrows indicate modified classification. Taxonomic categories are shown in bold (left).

Cryptosporidium has attributes that separates it from other coccidia including its small oocysts size, distinct attachment organelle, ability to reproduce two thin-walled and thick-walled oocysts, and insensitivity to anticoccidial drugs (Cacciò & Widmer, 2013; McDonald et al.,1990; Tenter et al., 2002; Xiao, Fayer, Ryan, & Upton, 2004). In addition, recent genetic analyses of *Cryptosporidium spp.* determined that the parasite is no longer a coccidian, but a gregarine because of its ability to multiply outside hosts (Clode, Koh, & Thompson, 2015); though this novel perception is highly debateable, thus warrants further investigation. Other key similarities

between *Cryptosporidium* and gregarines include: their large extracellular gamont stages and ability to modify their cell structure for environmental adaptation (Ryan, Paparini, Monis, & Hijjawi, 2016). Last, molecular evidence comparing the small-subunit (SSU) ribosomal ribonucleic acid (rRNA) of several gregarines and other Apicomplexan sequences determined that the phylogenetic affinity of *Cryptosporidium* was more closely related to gregarines than coccidia (Carreno, Matrin, & Barta, 1999).

1.2.2. Cryptosporidium Nomenclature and Host Specificity

Traditional nomenclature of *Cryptosporidium spp.* was based on host specificity. Newly discovered species were named after their host origin such as *C. bovis* from cattle (Fayer, Santin, & Xiao, 2005); *C. canis* from dogs (Fayer et al., 2001); *C. felis* from cats (Iseki, 1979); and *C. serpentis* from snakes etc. (Levine, 1980). Today, cross-transmission studies have revealed the heterogeneity of *Cryptosporidium spp.* in humans and animals. For example, *C. meleagridis* was traditionally linked to turkeys (Slavin,1955) and other bird species (Lindsay, Blagburn, & Sundermann, 1989). However, since its first reported case in humans (Morgan et al., 2000), there has been an increasing number of molecular investigations finding *C. meleagridis* responsible for anthropocentric infections (Akiyoshi, 2003; Chappell, 2011; Gatei, 2008). Thus, the practice of naming species after their host origin is no longer customary. Modern research now focuses on synonymizing the names of "new" *Cryptosporidium spp.*, in the same manner as *C. parvum* (Xiao et al., 2004).

Nomenclature has also been established for naming subtypes of *Cryptosporidium spp.*, which distinguishes intra-species variation based on sequencing of the 60 kDa glycoprotein (gp60) gene. The naming system begins with the family designation. For instance, the subtype families

of *C. hominis* are Ia, Ib, Ic, Id etc.; *C. parvum* are IIa, IIb, IIc, IId, etc.; *C. fayeri* are IVa, IVb, IVc, IVd etc. Second, the nomenclature provides the dominant trinucleotide sequences and the number of repeated copies. For example, an existing subtype of *C. hominis* is IeA12G3T3, where A12 denotes 12 copies of the TCA sequence; G3 denotes 3 copies of the TCG sequence; and T3 denotes 3 copies of the TCT sequence (Xiao & Feng, 2017). Last, a R1, R2, R3 etc. sometimes appear at the end of the naming system (e.g., *C. hominis* subtype IaA17R2) to distinguish the number of other repetitive sequences that may exist (Ryan, Fayer, & Xiao, 2014).

To date, scientists have classified at least 24 species/genotypes, as well as 65 subtype families of *Cryptosporidium*—with new discoveries continuously authenticated (Fayer, 2010; Xiao & Feng, 2017). Table 1.1. provides an updated list of the classified *Cryptosporidium spp*., along with their hosts. The data includes only formal and scientifically-named species, based on principals outlined by the International Code of Zoological Nomenclature (ICZN) (ICZN, 2016; Xiao et al., 2004).

Table 1.1.: ICZN Classified *Cryptosporidium Spp.* and Hosts

Species/ Genotypes	Subtype Family	Hosts	References
C. andersoni	N/A	*Bos taurus* (Cattle)[1] and other bovine animals; *Mus musculus* (Mice); *Ovis Aries* (Sheep); *Camelus* (Camels)	Lindsay et al. (2000)
C. baileyi	N/A	*Gallus gallus* (Chicken)[1]; *Homo sapiens* (Humans); *Coturnix coturnix* (Quails); *Struthio camelus* (Ostriches); *Anatidae* (Ducks)	Current, Upton and Haynes (1986)
C. bovis	N/A	*Bos taurus* (Cattle)[1] and other bovine animals; *Ovis Aries* (Sheep)	Fayer, Santin and Xiao (2005)
C. canis	N/A	*Canis lupus* (Dogs)[1]; *Homo sapiens* (Humans)	Fayer et al. (2001)

Chipmunk genotype I	XIVa	*Tamias* (Chipmunks)[1]; *Sciuridae* (Squirrels); *Mus musculus* (Mice); *Homo sapiens* (Humans)	As cited in Xiao and Feng (2017)
C. cuniculus	Va, Vb	*Oryctolagus cuniculus* (Rabbits)[1]; *Homo sapiens* (Humans)	Inman and Takeuchi (1979)
C. erinacei	XIIIa	*Erinaceinae* (Hedgehogs)[1]	Kváč et al. (2014)
Environmental sequence	N/A	N/A	Ruecker et al. (2011) (GenBank direct submission)
Ferret genotype	VIIIa	*Mustela putorius furo* (Ferret)	As cited in Xiao and Feng (2017)
C. fayeri	IVa, IVb, IVc, IVd, IVe, IVf	*Macropus rufus* (Kangaroos)[1]	Ryan, Power and Xiao (2008)
C. felis	N/A	*Felis catis* (Cats)[1]; *Homo sapiens* (Humans); *Bos taurus* (Cattle)	Iseki (1979)
C. galli	N/A	*Gallus gallus* (Chicken)[1]	Pavlásek (1999)
C. hominis	Ia, Ib, Id, Ie, If, Ig, Ih, Ii. Ij, Ik	*Homo sapiens* (Humans)[1]; *Bos taurus* (Cattle); *Ovis Aries* (Sheep); *Equidae* (Horses)	Morgan-Ryan et al. (2002)
Horse genotype	VIa, VIb, VIc	*Equus caballus* (Horses)[1]	As cited in Xiao and Feng (2017)
C. meleagridis	IIIa, IIIb, IIIc, IIId, IIIe, IIIf, IIIg, IIIh, IIIi, IIIj	*Meleagris gallopavo* (Turkey)[1] and other galliformes; *Homo sapiens* (Humans); *Psittaciformes* (Parrots)	Slavin (1955)
Mink genotype	Xa	*Neovison vison* (Minks)[1]; *Lutrinae* (Otters); *Mustela* (Ermines)	As cited in Xiao and Feng (2017)
C. molnari	N/A	*Sparus auratus*[1] *and Dicentrarchus labrax*[1] (Fishes)	Alvarez-Pellitero and Sitjà-Bobadilla (2002)
C. muris	N/A	*Mus musculus* (Mice)[1] and other rodents; *Homo sapiens* (Humans); *Bos taurus* (Cattle); *Capra aegagrus hircus* (Goat)	Tyzzer (1910)

C. nasorum	N/A	*Naso lituratus* (Fishes)[1]	Hoover, Hoerr, Carlton, Hinsman and Ferguson (1981)
Opossum genotype	XIa	*Didelphimorphia* (Opossum)[1]	As cited in Xiao and Feng (2017)
C. parvum	IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIi, IIk, IIl, IIm, IIn, IIo, IIp, IIq, IIr, IIs, IIt	*Mus musculus* (Mice)[1] and other rodents, *Homo sapiens* (Humans); *Bos Taurus* (Cattle); *Ovis Aries* (Sheep); *Sus* (Pigs); *Capra aegagrus hircus* (Goats); *Equidae* (Horses); *Cervidae* (Deer)	Tyzzer (1912)
C. saurophilum	N/A	*Squamata* (Lizards)[1]; *Elaphe guttata* (Snake)	Koudela and Modrý (1998)
C. scrofarum	N/A	*Sus* (Pigs)[1]	Kváč et al. (2013)
C. serpentis	N/A	*Elaphe guttata* (Snake)[1]; *Squamata* (Lizards)	Levine (1980)
Skunk genotype	XVIa	*Mephitidae* (Skunks)[1] and other rodents	As cited in Xiao and Feng (2017)
C. tyzzeri	IXa, IXb	*Mus musculus* (Mice)[1]	Ren et al. (2012)
C. ubiquitum	XIIa, XIIb, XIIc, XIId, XIIe, XIIf	*Cervidae* (Deer)[1] and other ruminants; rodents; *Homo sapiens* (Humans) and other primates	Fayer, Santín and Macarisin (2010)
C. varanii	N/A	*Varanus prasinus* (Monitor)[1]	Pavlásek et al. (1995)
C. viatorum	XVa	*Homo sapiens* (Humans) [1]	Elwin et al. (2012)
C. wrairi	VIIa	Guinea pig (Cavia porcellus)[1]	Vetterling, Jervis, Merril, and Sprinz (1971)
C. xiaoi	N/A	*Ovis Aries* (Sheep)[1]; *Capra aegagrus hircus* (Goat)	Fayer and Santín (2009)

Sources: Smith et al. (2007); Ghazy et al. (2015); Fayer (2010); Ren et al. (2012); Xiao and Feng (2017)

[1] signifies the first reported host of *Cryptosporidium spp.*

1.2.3. The Life Cycle of Cryptosporidium

Cryptosporidium initiates its life cycle as a sporulated oocysts. When the oocysts are ingested by a suitable host, excystation occurs and the genus sheds sporozoites (immature forms of the parasite) into the gastrointestinal system. The sporozoites invade the epithelial of the gastrointestinal tract and other tissues by attaching to enterocytes, which are cells of the intestinal lining. The sporozoites then transition to a growing trophozoite stage, which undergoes asexual reproduction by merogony, producing a type I meront, as well as 6-8 merozoites. The merozoites release back into the gastrointestinal track and either infect new enterocytes or transition into a type II meront, which is accompanied by 4 merozoites. All merozoites—developed from both type I meront and type II meront—infect new enterocytes forming a macrogamont (female) or a microgamont (male). The macrogamont and microgamont undergo sexual reproduction by gamogony, and thus form a zygote (Cacciò & Widmer, 2013; Leitch & He, 2012; Tenter et al., 2002). The zygote then asexually forms 4 internal sporozoites by sporogony, resulting in thin-walled and thick-walled oocysts.

Approximately, 20 % of oocysts are thin-walled and remain within the host. These oocysts rupture and re-infect the host by releasing additional sporozoites, which repeat the life cycle. The remaining oocysts are thick-walled and excrete back into the environment through fecal matter (Leitch & He, 2012; Ryan & Ray, 2004). Figure 1.2. shows the life cycle of *Cryptosporidium* including its monoxenous development, where all stages of reproduction (asexual and sexual) are occurring in a single host.

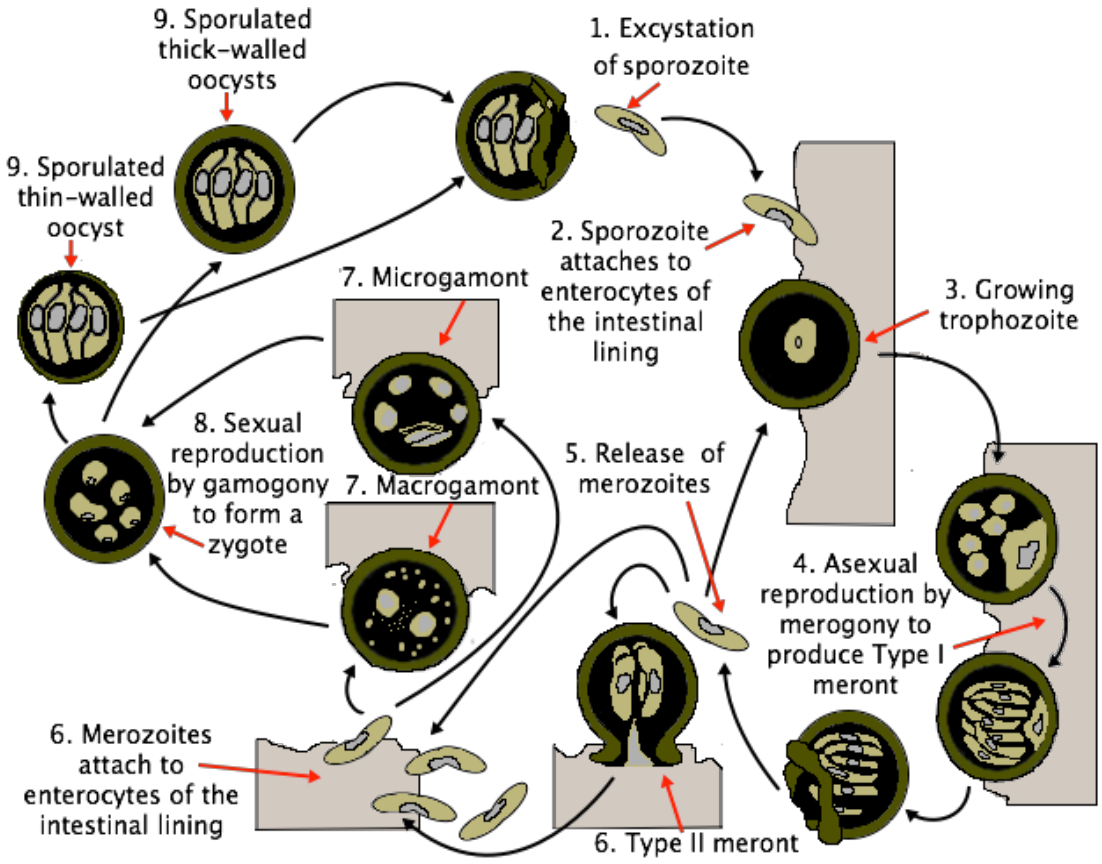

Figure 1.2.: The life cycle of *Cryptosporidium*. Adapted from sources: CDC (2017b); Bouzid, Hunter, Chalmers and Tyler (2013).

1.2.4. Cryptosporidium Transmission and Environmental Resilience

Cryptosporidium transmits via the oral-fecal route, and on rare occasions through emitted aerosols (Spickler, 2013). Figure 1.3. shows the various sources of *Cryptosporidium* transmission, based on global reports and research. It is evident that most sporadic outbreaks of cryptosporidiosis are traced to surface water, but infections have also been traced to groundwater and wastewater sources (Coupe et al., 2006; Corso et al., 2003; Daniels, Smith, Schmidt, Clasen, & Jenkins, 2016; D'antonio et al., 1985; Egorov et al., 2004; Fayer & Xiao, 2008; Gibson,

Stadterman, & Sykora, 1998; Hayes et al., 1989; Johnson, Pieniazek, & Rose, 1993; Karanis et al., 2006; Putignani & Menichella, 2010).

The spread of *Cryptosporidium* to surface water is largely due to animal droppings and manure applications to fertilized soils. Hofstra, Bouwman, Beusen and Medema (2013) developed a model-based inventory of global emissions and determined that oocysts transmitted from livestock to surface water is marginally higher than human emissions. The study however, did not consider transmission from wildlife populations—an area of research which warrants further investigation (Ziegler et al., 2007). For this reason, it can be expected that animal feces in soil collectively contain high volumes of oocysts compared to human feces.

Subsequent to heavy rainfall activity, water flows through and mixes with soil, producing leachate and runoff into surface water. Moreover, runoff occurs more frequently than leachate because soil provides filtration, hence most oocysts are retained in topsoil (Mawdsley, Brooks, & Merry, 1996). Soil-dwelling oocysts are highly resistant to environmental stressors. Walker, Montemagno and Jenkins (1998) reviewed studies on the survival and transport of *C. parvum* and determined that oocysts can survive in dry soils for several months, under cool, shaded conditions. In addition, the deactivation of oocysts in soil occurs at 6 months, 2 months, and 72 hours when exposed to 15°C, 25°C and 37°C, respectively (Fayer, Trout, & Jenkins, 1998). Moreover, feces shield oocysts from environmental pressures by reducing permeability of small molecules (Robertson, Campbell, & Smith, 1992). Therefore, oocysts can survive in various soils given their robust nature, and interaction with protective barriers.

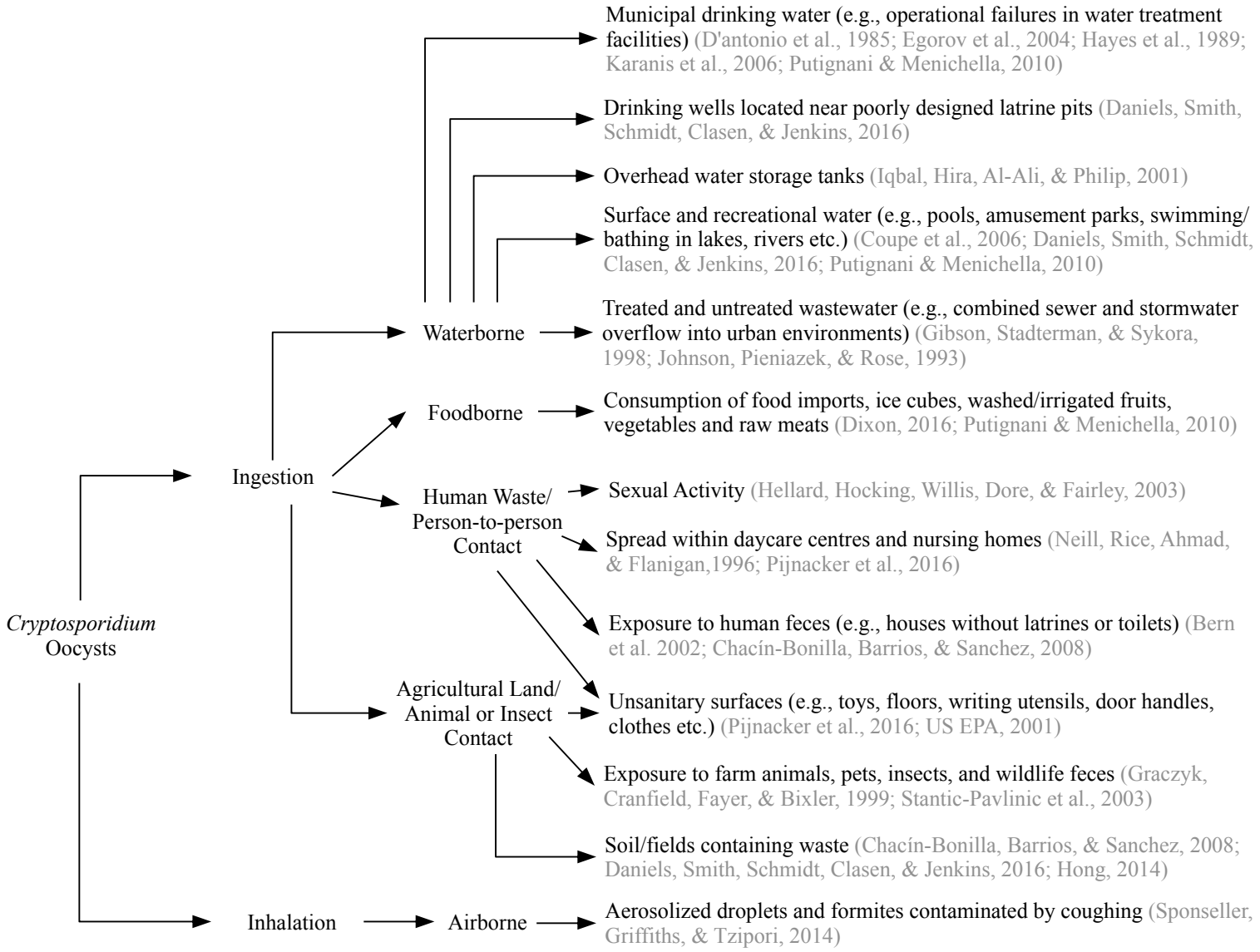

Figure 1.3.: Environmental transmission of *Cryptosporidium* based on global reports and research.

The four-main physiological and morphological characteristics that contribute to the environmental resilience of *Cryptosporidium* oocysts include:

1. **Tough outer shell:** resistant to extreme temperatures and most conventional disinfectants, including chlorination (at concentrations that are safe to work with).
2. **Small size:** permits transportation through filtration systems at water treatment plants.

3. **Infectivity:** ability to cause sickness in a broad range of hosts.
4. **Low-infectious dose:** requires 10 oocysts in humans and 50 oocysts in cattle to contract cryptosporidiosis, whereas a single bowel movement can release 10^7-10^8 oocysts (CDC, 2016a; DuPont et al.,1995; Moore et al., 2003).

1.3. Historical Overview

1.3.1. Early Detection of Cryptosporidium in Animals

The first publication that describes *Cryptosporidium* is accredited to an American physician and parasitologist by the name of Ernest Edward Tyzzer (1875-1965). Tyzzer described the parasite in the gastric glands of asymptomatic common mice using a simple light microscope. The parasite was noted to have various asexual and sexual life-cycle stages, and specialized organelles designed for the attachment to host cells. Also, spores were documented in the gastric glands of the mice, which was later described as indistinguishable or absent in the oocyst (Tyzzer, 1907). Based on these observations, Tyzzer coined the name *Cryptosporidium*, which translates to "hidden spore" (or Latin: "underground spore"). Shortly after, Tyzzer published two additional articles, which identified species of *Cryptosporidium* (also found in asymptomatic common mice). He named these species *C. muris* and *C. parvum* (Tyzzer, 1910; Tyzzer, 1912). Both publications provided further details about the physiology and life-cycle of the genus. However, *Cryptosporidium* research at the time lacked significance because the parasite was never known to cause sickness in humans nor animals.

By 1955, the species *C. meleagridis* was introduced after its discovery in turkeys. What made this discovery unique compared to prior detections was that the turkeys were experiencing symptoms of severe diarrhea. This marked the first reported case of *Cryptosporidium* as a

causative agent of illness and mortality (Slavin,1955). Nonetheless, *Cryptosporidium* research remained inconsequential, and from 1955 to 1970 there were only a few follow-up publications, in which *Cryptosporidium* was detected in a wide range of animals (Anderson, Duszynski, & Marquardt, 1968; Dubey & Pande, 1963; Jervish, Merrillt, & Sprinzh, 1966). Overall, *Cryptosporidium* research received little attention, and Tyzzer's initial three articles published in the early 1900s remained at the forefront of research, having defined most of what was known about the genus for the subsequent 43 years.

1.3.2. Increasing Economic and Public Health Interest in Cryptosporidiosis

During the 1970s and 1980s, *Cryptosporidium* was gaining economic significance. The parasite was frequently reported in symptomatic livestock including cattle, lamb, chicken and goats (Angus, Tzipori, & Gray, 1982; Barker & Carbonell, 1974; Nagy, Bozso, Pal, Nagy, & Sahibi, 1984; Snyder, Current, & Russek-Cohen, 1988; Tzipori, Campbell, Sherwood, Snodgrass, & Whitelaw, 1980; Widmer, 1998). Amoung this group, *Cryptosporidium* detection in cattle was most frequent. For example, *C. parvum* was detected in the small intestine of an 8-month-old calf that died after experiencing symptoms of dehydration and watery feces (Panciera, Thomassen, & Garner, 1971). Shortly after, many articles were published on the morbidity and mortality of dairy and beef calves due to cryptosporidiosis (Barker & Carbonell, 1974; Meuten, Van Kruiningen, & Lein, 1974; Morin, Lariviere, & Lather, 1976). Young calves were found most susceptible to the disease because unlike adult cattle, calves lack mature T cells (or T lymphocytes), which are white blood cells that actively participate in immune responses. Thus, *Cryptosporidium* was officially ruled as a probable factor of diarrhea in newborn calves (Pohlenz, Moon, Cheville, & Bemrick, 1978). This raised economic concerns in the cattle

industry regarding financial losses associated with cattle weight-loss, stunted-growth and to the lesser extent mortality. Cattle farmers were also worried about increasing veterinary and labour costs, including medicine and sanitary measures used to prevent the disease from spreading to other livestock and even pets (De Graaf, Vanopdenbosch, Ortega-Mora, Abbassi, & Peeters, 1999; Fayer & Ungar, 1986; House, 1978).

During 1970s and 1980s, *Cryptosporidium* also gained public health significance due to the increasing number of zoonotic incidences. For example, in 1976 two articles were published on the first human cases of cryptosporidiosis in an immunocompromised man and toddler (Meisel, Perera, Meligro, & Rubin, 1976; Nime, Burek, Page, Holscher, & Yardley, 1976). Both individuals lived on a cattle farm and had a dog in the house. Shortly after, many cases followed where human cryptosporidiosis was suspected to have been contracted through direct contact with animal feces (Current, 1985; Pitlik et al., 1983). There were also reports of *Cryptosporidium* spreading through person-to-person contact (especially in daycare centres), and through ingestion of fecal-contaminated food and water sources (CDC, 1984; Fayer & Ungar, 1986). By the 1980s, *Cryptosporidium* reached its peak in public health interest. During this era, several clinical studies revealed that the parasite was positively correlated with death in HIV and AIDS patients (Navin & Juranek, 1984; Pitlik et al., 1983; Tzipori & Widmer, 2008). This confirmed that *Cryptosporidium* was an opportunistic parasite, causing severe illness in immunocompromised individuals.

Although the heightened attention during the 1980s produced a plethora of publications, many reports mistaken *Cryptosporidium* for other pathogens. For example, sporocysts of *Sarcocystis spp.* were falsely perceived as *Cryptosporidium* oocysts because they are from the same Apicomplexa phylum, thus appear similar under the microscope (Levine, 1980). Nonetheless,

ultrastructural studies re-confirmed that *Cryptosporidium* has a distinct attachment organelle, which is an integral component of its classification (Xiao et al., 2004) (section 1.2.1.). Hence, from the 1990s to early 2000s, research pertaining to *Cryptosporidium* nomenclature and host specificity was further clarified, as microscopy and molecular techniques advanced (Cacciò & Widmer, 2013; Clode et al., 2015). Today, researchers continue to use these technologies to study the morphological characteristics of *Cryptosporidium spp.,* as well as to enhance the specificity and sensitivity of oocysts detection from environmental sources.

1.3.3. Significant Waterborne Outbreaks of Cryptosporidiosis

In July 1984, the first reported outbreak of waterborne cryptosporidiosis occurred in Braun Station, Texas. An estimated 2000 people—34% of the population—contracted cryptosporidiosis from a sewage-contaminated well. Based on these findings, D'antonio et al. (1985) recommended that *Cryptosporidium* should be added to the list of waterborne pathogens capable of causing outbreaks of gastroenteritis. Shortly after, in February 1987, another large-scale outbreak occurred in Western Georgia, where an estimated 13,000 residents—39% of the population—contracted cryptosporidiosis. The outbreak resulted from contaminated surface water, flowing through a water treatment facility during operational irregularities (Hayes et al., 1989). In both cases, drinking-water standards adhered to the World Health Organization (WHO)'s Guidelines for Drinking Water Quality, which focused on using *E. coli* as an indicator pathogen for monitoring *Cryptosporidium spp.* (Chalmers, 2012). Thus, post investigations determined that water treatment standards and the use of surrogate pathogens were insufficient in preventing *Cryptosporidium* from contaminating drinking water.

Similar conclusions were drawn following the April 1988 outbreak in Ayrshire, Scotland, which marked the first waterborne outbreak of cryptosporidiosis reported in the UK. Initial investigations diagnosed cryptosporidiosis in 27 people, and a follow up investigation determined that several hundred at the time experienced symptoms of diarrhea. The cause of the outbreak originated from the absence of fecal bacteria indicators, and operational failures during a time of heavy rainfall activity, which allowed animal slurry to contaminate the drinking water source (Smith et al., 1989). That same year leading into 1989, over 500 residents from Wiltshire and Oxfordshire, England, contracted cryptosporidiosis from treated water (Richardson et al., 1991). Evidently, these patterns continued into the early 1990s, when two large-scale outbreaks occurred in Oregon (Leland, McAnulty, Keene, & Stevens, 1993) and North-west England (Bridgman et al., 1995). Both outbreaks resulted from inadequate filtration and disinfectants (e.g., chlorination), which failed to prevent *Cryptosporidium* oocysts from bypassing treatment. Despite these systematic failures, it wasn't until the massive outbreak in Milwaukee, that prompted research and operational changes worldwide.

In the spring of 1993, the largest outbreak of *Cryptosporidium* in history occurred in the greater area of Milwaukee, Wisconsin. Based on telephone surveys and hospital records, 69 residents died and approximately 403,000 residents—26% of the 1.6 million population—experienced symptoms of the infection (Corso et al., 2003). At the time of the outbreak, the Milwaukee Water Works (MWW) ensured the region with potable water from Lake Michigan (Institute of medicine [IOM], 2009). Following an investigation, the Milwaukee outbreak was attributed to inadequate water treatment due to abnormal increases in turbidity between the months of March and April 1993. During this time, Milwaukee was experiencing greater-than-normal winds, precipitation, and snow melt (Eisenberg, Seto, Colford, Olivieri, & Spear, 1998). All of these factors contributed to the excessive runoff of water and debris containing *Cryptosporidium*

oocysts into Lake Michigan and subsequently to the Howard Avenue Water Treatment Plant (HWTP), which served one third of the population (Fox & Lytle, 1996; Gradus, 2014). The magnitude of the outbreak posed significant threat to the US water supply. The economic costs associated with medical care and productivity losses totaled $96.2 million (IOM, 2009). This emphasized on the need to establish better monitoring programs for *Cryptosporidium*. Thus, between 1993 and 2016, MWW invested a total of $459 million in water-related technologies and projects to ensure high-quality water and monitoring services (Water Research Foundation, 2016). Today, MWW is a champion for the development of an effective multiple-barrier strategy, which is currently modeled worldwide.

1.4. *Cryptosporidium* as a Global Burden

Cryptosporidium is globally widespread in developing and industrialized nations (Figure 1.4.). The greatest burden however, occurs in developing countries (CDC, 2017a), where most reported cases are associated with *C. parvum* and *C. hominis* species (Putignani, & Menichella, 2010), and children who suffer from famine and symptoms of moderate-to-severe diarrhea (Guerrant, 1997; Kotloff et al., 2012). In general, persistent diarrhea is the leading cause of death in children under the age of 5 in developing countries, accounting for 30-50% of childhood mortalities (Ochoa, Salazar-Lindo, & Cleary, 2004). Because *Cryptosporidium spp.* deplete the lining of the gastrointestinal track, the diarrheic disease is considered a contributing factor of environmental enteropathy—a condition derived from continuous exposure to fecal pathogens, which reduces the surface area of one's intestines, resulting in the inability to retain nutrients (Brown, Khatun, & Ahmed, 1981). Consequently, studies have found correlations between cryptosporidiosis and stunted growth in children from Uganda (Tumwine et al., 2003), Peru

(Checkley et al., 1998), Haiti (Kirkpatrick et al., 2006), and Bangladesh (Korpe et al., 2016). Other correlations include: cognitive impairment, gut microbiome perturbation, malnutrition, reduced vaccination efficacy and susceptibility to other diseases (Agnew et al., 1998; Alcantara et al., 2003; Guerrant, 1997; Guerrant et al., 1998; Lang & MAL-ED Network Investigators, 2015; Shoultz, de Hostos, & Choy, 2016).

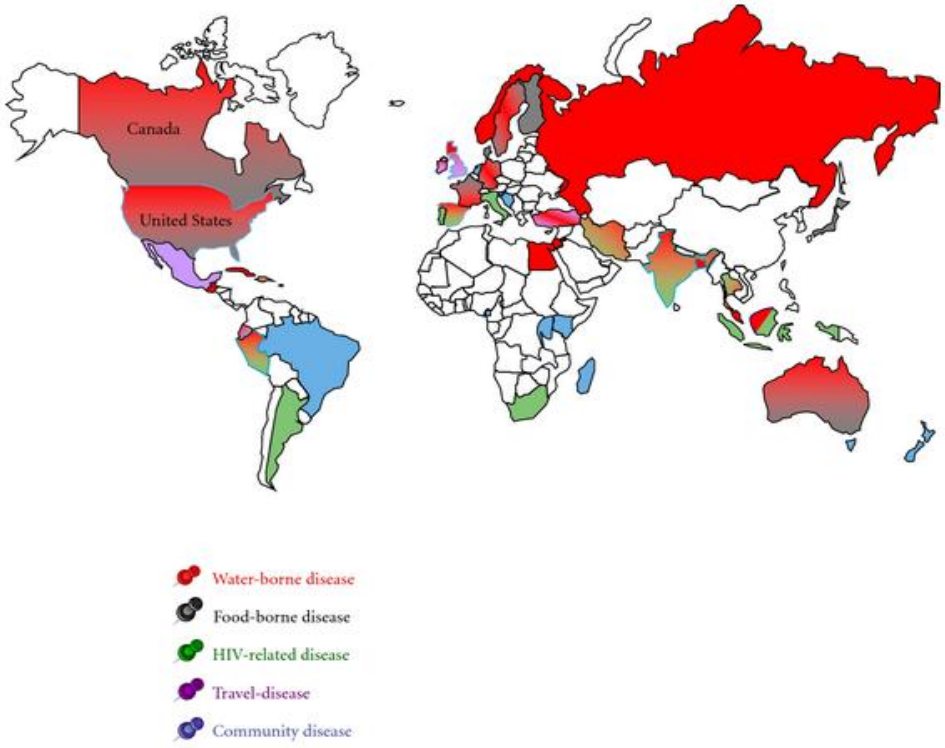

Figure 1.4.: Global sporadic outbreaks of cryptosporidiosis between 1998 and 2008. Source: Putignani & Menichella (2010).

While other pathogens cause moderate-to-severe diarrhea in children, C*ryptosporidium* is amoung the most prevalent in developing countries. For example, the *Global Enteric Multicenter Study* (GEMS) (2007-2011) was conducted in 7 diverse and highly-burden sites in Asia and Africa, including Gambia, Kenya, Mali, Mozambique, Bangladesh, India and Pakistan. The

comprehensive study assessed moderate-to-severe diarrhea in approximately 22,000 children under the age of 5 and concluded that cryptosporidiosis is the second largest cause of diarrheal death in infants (second to Rotavirous) (Kotloff et al., 2012). Similar conclusions were drawn from the *Malnutrition and the Consequences for Child Health and Development* (Mal-ED) study (2009-2014), where enteric pathogens were analyzed in over 2100 children from Sub-Saharan Africa, Asia, and South America including South Africa, Tanzania, India, Bangladesh, Pakistan, Nepal, Brazil and Peru. The study concluded that *Cryptosporidium* was ranked 5 of 22 detected pathogens in children under the age of 24-months (Platts-Mills et al., 2015). Overall, the large-scale GEMS and Mal-ED investigations demonstrate that *Cryptosporidium* plays an integral role in causing illness and mortality in children of low-income countries.

Apart from childhood infections, cryptosporidiosis is a burden amoung the general population and visiting travelers of developing countries. For example, Russian and Bulgarian drinking waters have tested positive for *Cryptosporidium* from municipal tap and well sources (Karanis et al., 2006). Furthermore, cryptosporidiosis has been diagnosed in returning travelers who visited Russia (Jokipii, Pohjola, & Jokipii, 1983), the Caribbean (Ma, Kaufman, Helmick, d'Souza, & Navin, 1985), Egypt, Mauritius (Gatti, Cevini, Bruno, Bernuzzi, & Scaglia, 1993), Pakistan (Flegg, 1987) and Mexico (Nair et al., 2008). As such, the WHO added cryptosporidiosis to its Neglected Diseases Initiative database in 2004. The database consists of parasitic, bacterial and viral diseases that occur mainly in developing countries, and are connected to climate, poverty and inaccessible services (Savioli, Smith, & Thompson, 2006). Cryptosporidiosis in particular, is often linked to remote and rural communities, urban overcrowded cities and natural disaster zones (Damiani et al., 2013; Putignani, & Menichella, 2010; Snelling et al., 2007). Furthermore, food and poor sanitation are typical sources of enteric-pathogen exposure in developing

countries; however, due to inaccessible technologies and healthcare infrastructure, these sources are under reported in relation to cryptosporidiosis (Ashbolt, 2004).

Last, cryptosporidiosis is a burden in industrialized countries. Putignani and Menichella (2010) composed a global review, which investigated 71 sporadic outbreaks of cryptosporidiosis reported between 1998 and 2008. The study determined that most outbreaks were waterborne (56.3%), and infected both adults and children. In addition, most outbreaks were concentrated in developed countries, including the US, Canada, Australia, UK and Ireland. Notably, these findings do not reflect the global underreporting of cryptosporidiosis; however, they do disclose that the parasite is a burden even in high-income countries. Cryptosporidiosis in developed countries are often associated with small drinking water communities, which sometimes employ primitive water treatment processes that are incapable of eradicating *Cryptosporidium* oocysts or have regular failures that are not addressed (Pons et al., 2015). In addition, waterborne outbreaks are often associated with recreational water-use (e.g., amusement parks and swimming pools) (Putignani & Menichella, 2010). Conversely, over the past 30 years, improved technologies, water-quality management practices, and monitoring of recreational and drinking water catchment areas have resulted in reduced cryptosporidiosis infections in industrialized nations (Bridge et al., 2010; Lake et al., 2007). Unfortunately, the burden and consequences of cryptosporidiosis falls heavily on developing countries in the absence of such interventions.

1.5. *Cryptosporidium* in the Caribbean

Only a few publications have investigated *Cryptosporidium* in the Caribbean. All studies were conducted in the capital cities and surrounding regions of Cuba, Jamaica and Haiti. Most investigations focused on the clinical manifestation of cryptosporidiosis in human populations.

More recently, research has shifted to focus on existing species/genotypes and subtypes of *Cryptosporidium*, along with surface and groundwater prevalence; however, this data is limited.

The first publication dates back to Cuba in 1989. Delfin, Sanjurjo, Findlay and Gordeeva (1989) collected fecal specimens from 200 children—ages 2 months to 4 years—experiencing diarrhea at the Children's Hospital of Havana City. Various enteric pathogens were assessed while using a formol-ether sedimentation method, followed by modified Ziehl-Neelsen microscopy. *Cryptosporidium* was identified in 8% of patients, which was the second most common pathogen detected. Moreover, *Cryptosporidium* was found most frequent in the youngest patients. Subsequent investigations in Cuba confirmed this correlation as *Cryptosporidium* infections were identified amoung young children, from pre-school to 8 years, in the provinces of Ciego de Ávila and Havana (Núñez et al., 2003a; Núñez et al., 2003b; Pelayo et al., 2015). Similarly, Lindo et al. (1998) was the first pilot study to investigate cryptosporidiosis in Jamaica. A total of 328 stool samples from patients less than 1 to 81 years of age were collected from the University Hospital of the West Indies (UHWI) in Kingston. The study determined that 4% of samples tested positive for *C. parvum*, of which highest prevalence was observed amoung children under the age of 2. Also, *C. parvum* infections occurred in 3 adults who had HIV.

In Haiti, *Cryptosporidium* research was becoming increasingly popular, especially after the January 2010 earthquake in the capital city, Port-au-Prince (Damiani et al., 2013). The aftermath of earthquakes is known to displace populations and change environmental conditions, resulting in communal vulnerability to existing pathogens, including *Cryptosporidium* (Devane, Moriarty, Wood, Webster-Brown, & Gilpin, 2014; Marahatta, 2015). In Haiti, *Cryptosporidium* research is also relevant because the parasite was previously deemed the most frequent cause of diarrhea in the country—responsible for 17% of acute diarrhea in infants under the age of 2, and 30% of

severe diarrhea in HIV-infected patients (Pape et al., 1987; Pape & Johnson, 1993). High correlations have also been linked to low socio-economic conditions and over crowdedness in large Haitian cities (Raccurt et al., 2006).

In terms of *Cryptosporidium* species and genotypes, Haiti was the first to investigate this in the Caribbean. Raccurt et al. (2006) analyzed 1529 stool samples from patients with diarrhoea at the GHESKIO Centres and the University Hospital in Port-au-Prince. Approximately, 10% of adults and children tested positive for cryptosporidiosis via modified Ziehl-Neelsen microscopy. Amoung this group, a genotyping tool, referred to as restriction fragment length polymorphism (RFLP)-PCR was used to determine a species population consisting of *C. hominis* (59%), *C. parvum* (38%) and *C. felis* (3%) from 69 isolates. The data confirmed a high prevalence of *C. hominis* in young patients, whereas immunocompromised patients were equally infected by both human and animal genotypes. That same year, Ngouanesavanh et al. (2006) investigated *Cryptosporidium spp.* population in 49 Haitian patients, also from the GHESKIO Centres and University hospital of Haiti. *C. hominis* and *C. parvum* were detected in 3 and 2 patients, respectively, which was interpreted as epidemic clonality. In other words, the low genomic diversity suggests that the same or similar isolates of *Cryptosporidium* were continuously involved in either geographical or temporal outbreaks in Haiti.

In Jamaica, advanced molecular research was performed by Gatei et al. (2008). Stool samples were collected from HIV-infected adults from various hospitals in Kingston, Jamaica. A total of 35 individuals tested positive for *Cryptosporidium*. PCR and gene sequencing techniques were used to determine a species population of *C. hominis* (69%), *C. parvum* (20%), *C. felis* (3%), *C. canis* (3%), and a *C. felis and C. hominis* (3%) coinfection. In addition, sequencing of the gp60 gene determined that *C. hominis* specimens were subtypes IbA10G2 (88%) and IeA12G3T3

(12%). Whereas, *C. parvum* specimens were subtype IIcA5G3d (Gatei et al., 2008), which is generally associated with patients who experience more severe symptoms of cryptosporidiosis (Iqbal, Lim, Al Mahdy, Dixon, & Surin, 2012). In Cuba, gp60 analysis of *Cryptosporidium* infections was also performed. Stool samples from 28 children (ages 2–8 years) were assessed, of which 10 *C. hominis* subtypes were determined, but no *C. parvum*. The subtypes identified were from the Ia, Ib and Id families, demonstrating considerable diversity.

Finally, research pertaining to *Cryptosporidium* detection from water sources is very limited in the Caribbean. Damiani et al. (2013) is the only available publication to have investigated the prevalence of *Cryptosporidium spp.* in surface and groundwater sources. A total of 16 water samples were collected from Port-au-Prince and Cap Haïtien, Haiti. *Cryptosporidium* was detected in 15 samples via microscopy and PCR, but only *C. parvum* was sequenced in all positive samples. The study noted that it was difficult to determine mixed populations of *Cryptosporidium spp.* from environmental samples, possibly due to inhibitory factors and the lack of DNA present.

1.6. Methods for Detecting and Genotyping *Cryptosporidium spp.* from Environmental Samples

1.6.1. Polymerase Chain Reaction (PCR) and Gene Sequencing Applications Proceeding the 1993 Cryptosporidium Outbreak in Milwaukee

One of the most influential achievements to rise from the Milwaukee outbreak was the application of PCR for rapid detection and gene sequencing of *Cryptosporidium spp*. Johnson et al. (1995) was the first to publish a conventional PCR protocol, which tested 14 preserved water-samples from the Milwaukee outbreak. The protocol was developed to improve the specificity of

oocysts detection in drinking-water sources. Briefly, samples were centrifuged, washed, and subjected to a novel six-cycle freezing and thawing step, to extract RNA from the interior part of the oocysts, targeting the 18S rRNA gene prior to running PCR. Once completed, the study confirmed the presence of *Cryptosporidium* in 21.4% (*n*=3) of Milwaukee's samples, which was initially confirmed via microscopy. The practicality of PCR was found comparable to microscopic methods, as it could simultaneously deliver rapid analysis of many samples at a relatively low cost (Johnson et al., 1993; Morgan & Thompson, 1998; Rochelle, De Leon, Stewart, & Wolfe, 1997a). Therefore, Johnson et al. (1995) developed a PCR protocol following the Milwaukee outbreak, which offered a new way to confirm oocysts detection in water. Since then, many researchers adopted and improved the efficiency of PCR, specifically for *Cryptosporidium* detection and gene sequencing from water sources (DiGiorgio, Gonzalez, & Huitt, 2002; Jiang, Alderisio, Singh, & Xiao, 2005; Li et al., 2012; Morgan, Constantine, Forbes, & Thompson, 1997; Rochelle, De Leon, Stewart, & Wolfe, 1997b; Stinear, Matusan, Hines, & Sandery, 1996; Sulaiman, 1998; Xiao, Alderisio, Limor, Royer, & Lal, 2000; Zhou, Singh, Jiang, & Xiao, 2003).

Over the ensuring decades, PCR technology developed, and researchers began to use variations of the technique to identify species and genotypes of *Cryptosporidium* isolates from archived Milwaukee samples. This laid the foundation for source-water tracking approaches tailored specifically to monitor the epidemiology of *Cryptosporidium.* For instance, the outbreak was initially suspected to be caused by a cattle genotype of *C. parvum*—a species that infects both humans and animals (Gradus, 2014; Mac Kenzie et al.,1994). Dairy cattle are major reservoirs of *C. parvum* (O'Handley et al., 1999), and in 1993, Wisconsin was the second largest dairy-producing state in the country (United States Department of Agriculture [USDA], 2017). Human wastewater and local slaughterhouses were also suspected to be potential sources of the outbreak

(Mac Kenzie et al, 1994). However, in 1998, retrospective analysis of clinical isolates preserved from the Milwaukee outbreak confirmed that most genotypes were in fact anthropocentric (Sulaiman, 1998).

The human-type isolates were confirmed by Sulaiman (1998). The study used RFLP-PCR to determine inter-species variations of *Cryptosporidium*. The technique could differentiate human from animal isolates, which verified that the genotypes from the outbreak were mostly human. Nearly a decade after the outbreak, Zhou, Singh, Jiang and Xiao (2003) also used RFLP-PCR to analyze Milwaukee wastewater, which confirmed similar results. In the study, *C. parvum* (human genotype-1)—now known as *C. hominis*—was found indistinguishable from the outbreak isolates, which indicated there had been continuous transmissions of human cryptosporidiosis in the city. In addition, the study used nested-PCR to validate these findings, where three or more sets of primers were used to identify *Cryptosporidium spp.* rather than one. Today, RFLP-PCR and nested-PCR are common applications for *Cryptosporidium* detection and genotyping from water sources (Mayer & Palmer, 1996; Nichols, Campbell, & Smith, 2003; Nikaeen, Mesdaghinia, Tehrani, Rezaeian, & Makimura, 2005; Sturbaum et al., 2001).

Further explorations of PCR and gene sequencing occurred post 1993. For example, reverse transcription-PCR (RT-PCR) of the mRNA hsp70 gene is used to determine viability of oocysts detected from water (Hallier-Soulier & Guillot, 2003; Rochelle et al., 1997a; Stinear et al., 1996). Cell culture-PCR (CC-PCR) is used for *in vitro* culturing of *Cryptosporidium* within HCT-8 cells to determine infectivity (Di Giovanni et al., 1999; Keegan, Fanok, Monis, & Saint, 2003; Le Chevallier et al., 2003; Rochelle et al., 1997a). Gene sequencing and phylogenetic analysis of PCR products are used to confirm positive detections, as well as to source track *Cryptosporidium spp.* to their origin (Prystajecky, Huck, Schreier, & Isaac-Renton, 2014; Yang

et al., 2008). Moreover, sequencing of the gp60 gene is used to determine the subtypes of *Cryptosporidium spp.*—a common practice that assess the genetic diversity and transmission dynamics of the parasite (Stensvold, Beser, Axén, & Lebbad, 2014). Last, PCR applications collectively, are used to evaluate existing primers that target different *Cryptosporidium* genes or different *loci* of the same genes (Guy, Payment, Krull, & Horgen, 2003; Rochelle et al., 1997a). Overall, these studies continue to build on existing databases, which have produced many new primers that are species specific. Therefore, the application of PCR for *Cryptosporidium* detection was first put in an epidemiological context when determining the source of contamination of the notorious Milwaukee outbreak. Since then, the same PCR protocols have been referenced globally in many *Cryptosporidium* studies, including surface water, storm water and wastewater applications (Li et al., 2012; Mayer & Palmer, 1996; Ruecker et al., 2005; Xiao et al., 2000; Yang et al., 2008).

Although PCR and gene sequencing applications are useful for identifying *Cryptosporidium spp*., many researchers have uncovered limitations pertaining to specificity and sensitivity. Sulaiman, Xiao and Lal (1999) repeated past PCR protocols used to detect *C. parvum* between 1994 and 1998. The study sequenced the DNA of PCR products and revealed that the primers used in many protocols amplified the DNA of closely related *Eimeria* species, producing false-positives. In addition, Sturbaum et al. (2001) used a nested RFLP-PCR technique to evaluate its sensitivity for oocysts detection and genotyping from water sources. The study determined an average detection rate of 38% for a single oocyst, whereas greater than 90% for five or more oocysts. Furthermore, researchers observed reduced sensitivity of PCR applications when analyzing oocysts from environmental samples due to high-level of inhibitors and low DNA yield (Adamska, Leonska-Duniec, Sawczuk, Maciejewska, & Skotarczak, 2012; DiGiorgio et al., 2002; IOM, 2009). Overall, PCR amplifications and sequencing techniques are limited due to

non-specific primers, reduced sensitivity of samples that contain low number of oocysts (DNA), and the presence of inhibitors.

1.6.2. US EPA Method 1623: Filtration/Immunomagnetic Separation (IMS)/ Immunofluorescence Assay (IFA) Microscopy

Given the uncertainties of PCR, microscopic methods have been standardized to reduce the overall subjectivity of *Cryptosporidium* oocysts identification. The US Environmental Protection Agency (EPA) Method 1623 is one of the most widely accepted protocol used to detect *Cryptosporidium* from aqueous matrixes via Immunomagnetic Separation (IMS)-immunofluorescence assay (IFA) microscopy. The mandated protocol was developed following the Milwaukee outbreak, and it is based on laboratory recommendations convened by a panel of experts.

The method involves four main steps: 1) raw water is pumped from the site; 2) samples are concentrated via filtration and centrifugation; 3) IMS isolates any oocysts contained within the samples from debris and non-targeted microorganisms; and 4) enumeration is performed via IFA microscopy, and further characterization is performed by 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy as shown in Figure 1.5. (US EPA, 2005).

Apart from microscopic analysis, the IMS component of Method 1623 is used to remove inhibitors prior to running PCR (Jiang et al., 2005; Sturbaum et al., 2002). Researchers also perform the method for screening, as well as for verification of PCR positive detections (Di

Giovanni, 1999; Hallier-Soulier & Guillot, 2003; Krometis, Characklis, & Sobsey, 2009; Lowery, 2001; Sturbaum et al., 2002).

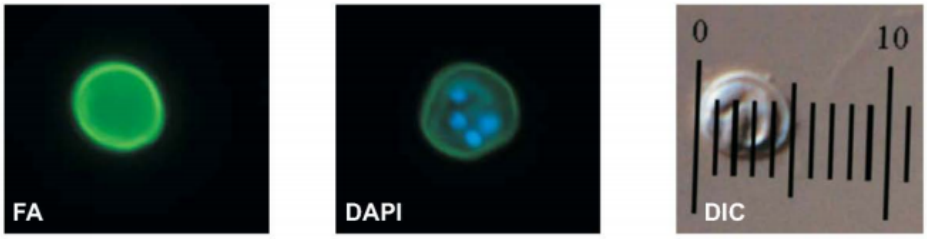

Figure 1.5.: *Cryptosporidium* oocysts under IFA, DAPI and DIC microscopy. Source: US EPA (2012).

Overall, Method 1623 has demonstrated high oocyst-recovery rates and improved specificity. Roser, Ashbolt, Ongerth, & Vesey (2002) designed a proficiency test, where 5 experienced laboratories used Method 1623 to recover seeded oocysts from water. Satisfactory performances obtained oocyst-recovery rates between 45 and 81%. These results are substantially higher than the earlier EPA Information Collection Rule (ICR) method, which typically obtains lower recovery rates between 7 and 11% (Clancy, Gollnitz, & Tabib, 1994; Hsu, Huang, & Yeh, 2002). Furthermore, Method 1623 has shown to improve the specificity of oocysts identification by providing specific guidelines to assert positive detections as outline in section 3.2.3. This reduces the overall subjectivity of results.

In contrast, the downfall of Method 1623 is its high overhead costs associated with equipment and labour as demonstrated in Table F.I., Appendix F. Also, the method requires a high degree of analytical expertise to properly assert positive detections under microscopy (Clancy et al., 1994; Jakubowski et al., 1996; Le Chevallier, Norton, Siegel, & Abbaszadegan, 1995; Rochelle et al., 1997a). This is mainly because the monoclonal antibody used for staining sometimes cross-

reacts with species of algae (Waterborne Inc., 2010). Moreover, naturally occurring factors such as turbidity and low oocysts levels, generate inconsistent recovery rates (DiGiorgio et al., 2002). All of these factors highlight the difficulty of using the method to determine oocysts concentration and make predictions about the environment. The only way to obtain a comprehensive representation of oocysts in natural waters is to filter multiple samples at a single site during different times of the day. Performing this step requires specialized equipment, which is too expensive to adopt in most countries for regular surveillance (Hsu et al., 2002).

1.6.3. Modified Acid-Fast (MAF) Staining: White Light Microscopy

Prior to IFA microscopy, the gold standard for *Cryptosporidium* oocysts detection from fecal samples was MAF staining, in which oocysts are stained a pinkish-red colour, contrasting to a dark green or blue counter stain (Figure 1.6.). Kinyoun's Carbol Fuchsin (KCF) is a popular stain used for the procedure, developed in Paik and Suggs (as cited in Ma & Soave, 1983). Since then, the CDC has developed its own MAF protocol, which incorporates KCF for identifying *Cryptosporidium* oocysts from stool samples. An alternative stain is Ziehl-Neelsen; although, KCF does not require a pre-heating step (CDC, 2016b), thus it is presumed a faster diagnostic tool.

In terms of efficiency, MAF staining is considered less specific and sensitive than IFA (CDC, 2016c). Nonetheless, MAF remains the most common diagnostic tool used for *Cryptosporidium* detection in fecal samples. This is because MAF reagents are inexpensive and do not require specialized equipment. Arguably, if MAF is executed appropriately (e.g., sufficient staining time and analysis), then it can perform just as well or even better than IFA (Cole, 1997).

In comparison to non-microscope methods such as ELISA and PCR, MAF is less expensive; however, its labourious and consumes a lot of time (Rekha, Puttalakshmamma, & D'Souza, 2016). In addition, MAF is prone to error since *Cryptosporidium* oocysts are small and can be easily confused with artifacts (Tahvildar-Biderouni & Salehi, 2014). It is also possible to mistaken *Cryptosporidium* oocysts for other coccidian species including *Cystoisospora, Cyclospora*, yeast and mould. These species are generally larger than *Cryptosporidium*, but they appear similar and often take up the same pinkish red colour (Rekha et al., 2016). It is also possible that some oocysts do not stain due to over exposure to decolourizer. Overall, MAF requires microscopic expertise, thus it is more prone to error than ELISA and PCR methods.

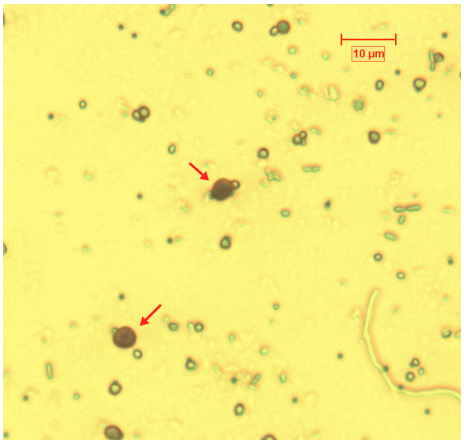

Figure 1.6.: MAF stained, positive control *Cryptosporidium* oocysts from cattle feces captured under white light microscopy. The Ward's ® Chemistry Acid-Fast Stain Kit was used. The carbol fuchsin stained two oocysts a deep pink colour. Oocysts have a well-formed wall with diameter of approximately 4-5 µm. Magnification, 100X with immersion oil. Image curtesy of Simmoy Noble, Ryerson University.

1.6.4. Enzyme-linked Immunosorbent Assay (ELISA) Test

ELISA is an immunoassay, typically used for clinical and veterinary detection of *Cryptosporidium* from stool samples. The technique uses either antigens or antibodies to react

with the presence of the parasite. This is performed by transferring stool specimens into 96-microwell plates that are coated with the species' antigens or anti-*Cryptosporidium* antibodies. The presence of *Cryptosporidium* is determined through a colour change when the antigens/antibodies from stool react to the coated surfaces of the plastic wells (Diagnostic Automation, Inc., 2003; Vohra, Sharma, & Chaudary, 2012).

Utilization of ELISA offers an accurate, fast and easy-to-read method. Studies have determined that the sensitivity of ELISA is approximately 87-93%, while the specificity is 95-99% (Ghoshal, Jain, Dey, & Ranjan, 2018; Rosenblatt and Sloan, 1993). Moreover, ELISA methods are easy to perform because they do not require observation of intact oocysts. Thus, proficiency tests have shown to produce results comparable to that of experienced microscopists (Diagnostic Automation, Inc., 2003)

While ELISA has proven to be a reliable method, a major drawback is its high cost (Vohra et al., 2012). Also, like many other antigen-based diagnostic tests, ELISA fails to differentiate disease carriers who are shedding oocysts, from those who have recently recovered. For example, ELISA kits designed to detect antibodies specific to *Cryptosporidium spp.* have shown positive for patients who recently recovered from confirmed infections, but still have detectable antibodies in their stool (Campbell & Current, 1983). Furthermore, immunoassays that target *Cryptosporidium* antigens have shown to produce false-negatives due to low parasite density in the fecal specimens (Vohra et al., 2012).

1.7. Specific Water Parameters that Influence the Morphology, Survival and Dispersion of *Cryptosporidium* Oocysts

Various water parameters including pH, dissolved oxygen (DO), *E. coli*/fecal coliforms, dissolved organic carbon (DOC), temperature, water hardness and salinity, influence the survival and dispersion of *Cryptosporidium* oocysts. These parameters either shield or change the morphological characteristics of oocysts, as well as produce microclimates in which they flourish. Here, specific details about how each parameter influences the longevity of oocysts, and whether they can be used to indicate risk of contamination is provided.

pH is used to measure the acidity and basicity of water. The reaction between water molecules and other compounds result in imbalances, where water becomes more acidic (more H+) or basic (more OH-) (Behar, 1997). In relation to *Cryptosporidium*, pH influences the surface charge of oocysts. Naturally, oocysts have a neutral surface charge when water is maintained at a neutral pH level. However, the presence of natural organic matter (NOM) in water lowers pH, resulting in a negative surface charge of oocysts. This in turn effects their electrostatic binding capacity to other material present in water (Dai & Hozalski, 2002). In addition, studies have investigated the correlation between pH and oocysts viability and determined that the number of viable oocysts slightly decreases at pH levels < 4 or >11 (Jenkins, Bowman, & Ghiorse, 1998; Kniel et al., 2003). In contrast, major viability losses occur at pH ≤ 1 or ≥ 13 (Campbell, Robertson, & Smith, 1992). Therefore, under extreme acidic and basic conditions, pH significantly decreases the number of viable oocysts. Since freshwater and saltwater typically maintain a pH between 6 and 8.1 (National Geographic Society, 2017; Peacock et al., 2004), pH is not an effective parameter used to predict the risk of *Cryptosporidium* contamination from environmental sources.

DO measures the amount of oxygen gas in water. In general, high DO levels are consistent with healthy ecosystems; whereas low DO levels are consistent with oxygen-consuming coliforms and organic waste, including sewage and oil biproducts, which are locally referred to as

'dundar'. Sente et al. (2016) collected water from the Queen Elizabeth Protected Area, Uganda, and observed a strong negative correlation ($p < 0.05$) between DO and the presence of *Cryptosporidium*. Similarly, Hogan et al. (2012) analyzed fecal and water samples from California wetlands and observed the same negative correlation between DO and protozoal counts ($p < 0.0001$), which included *Cryptosporidium spp*. Thus, low DO concentrations in water indicates the presence of excess fecal coliforms and organic waste. Since *Cryptosporidium spp*. derive from the same sources (e.g., sewage and agricultural runoff), DO could potentially be used to predict oocysts contamination.

For the same reasoning, *E. coli* and fecal coliforms are also potential indicators; however, numerous studies have shown that these parameters sometimes fail to correlate with *Cryptosporidium* (Harwood et al., 2005; Lalancette et al., 2014). Therefore, monitoring a selective group of organisms rather than a single surrogate pathogen, is more likely to predict the presence of oocysts in water.

DOC can be defined as the total organic material dissolved in water. DOC is calculated by measuring the amount of organic matter that passes through a filter. Water treatment facilities have found that low DOC correlates with the log reduction of *Cryptosporidium* oocysts (Considine, Dixon, & Drummond, 2002; Xagoraraki & Harrington, 2004). This is due to two possible factors. First, the presence of DOC causes the surface-layer proteins of oocysts to collapse, resulting in increased adhesion to organic matter (Plummer, Edzwald, & Kelley, 1995). Second, DOC inhibits the inactivation of oocysts by UV light. Therefore, high DOC content (e.g., algae) prevents the penetration of the sun's rays, which normally would denature oocysts (King, Hoefel, Daminato, Fanok, & Monis, 2008). All in all, DOC influences the presence of *Cryptosporidium* oocysts in water by enhancing their adhesion to organic material, which in turn

shields them from the sun's UV rays. For this reason, DOC may be a sufficient water parameter used to predict *Cryptosporidium* contamination.

Water temperature is influenced by air temperature, sunlight, inflow of groundwater, terrestrial runoff and turbidity. Current research suggests that oocysts maintain infectivity for at least 3 months when stored in water temperatures between 4 and 15°C (King, Keegan, Monis, & Saint, 2005). At 20°C, *Cryptosporidium* oocysts can survive for about 6 months, while at a slightly higher temperature of 25°C, oocysts inactivate after 3 months (Fayer et al., 1998). This means that the ideal temperature for *Cryptosporidium spp.* to thrive is approximately 20°C. Under extreme temperatures however, oocysts lose their infectivity. For example, in hot water, inactivation of oocysts occurs at 71.7°C (after 15 seconds) (Harp, Fayer, Pesch, & Jackson, 1996), whereas in frozen water, oocysts inactivate at -70°C and -20°C at 1 and 24 hours, respectively (Fayer & Nerad, 1996). Therefore, water temperatures influence the survival of *Cryptosporidium* oocysts, enabling them to thrive under optimal temperatures, and deactivate in extreme hot or cold water. Depending on climate, water temperature can be used to predict the seasons, in which oocysts are expected to be most prevalent.

Hard water contains high concentrations of calcium and magnesium, which dissolves as water percolates limestone ($CaCO_3$ or $MgCO_3$). Salinity also contributes to water hardness. For example, the total water hardness of freshwater is generally between 10 mg/l to 250 mg/l (0.01 ppt to 0.25 ppt) as $CaCO_3$; whereas the hardness of seawater is usually greater than 6000 mg/l (6 ppt) (Vernier Software & Technology, n.d.). Studies have found that *Cryptosporidium* oocysts can persist in hard waters for several months. Modini, A. Pizarro, M. Pizarro and Zerbatto (2016) investigated the viability of oocysts in artificial saltwater (salinity: 35 ppt) maintained at 20°C. Through *in vitro* excystation, it was determined that a large proportion of oocysts was viable

(28.6±9.79%) in seawater after 16 weeks. A slightly lower longevity was determined in seawater by Fayer, Graczyk, Lewis, Trout, & Farley (1998). In the study, *Cryptosporidium* oocysts was inoculated in artificial seawater (salinity: 10-30 ppt) maintained at 20°C, then bio-assayed in 5-to-7-day old mice to assess viability. It was determined that oocysts were viable at salinities of 0-10 ppt, 20 ppt and 30 ppt for 12 weeks, 4 weeks, and 2 weeks, respectively. Therefore, water hardness in freshwater is usually an insignificant indicator of *Cryptosporidium*; however, in coastal region and islands, where there is an influx of brackish water from the sea, water hardness may elevate to >30 ppt. In such cases, water hardness may be an important parameter to measure for predicting *Cryptosporidium* viability.

1.8. *Cryptosporidium*-Biofilm Association

1.8.1. Aquatic Biofilms

Aquatic biofilms also influence the survival and transport of *Cryptosporidium* oocysts. A biofilm is an aggregation of microorganisms, oriented in a highly efficient and stable system. Biofilms settle in water columns and adhere to many surfaces such as rocks and sediments of stream beds (Langmark, Storey, Ashbolt, & Stenstrom, 2005; Stoodley, Sauer, Davies, & Costerton, 2002). The biofilm structure is very complex, usually consisting of bacteria embedded in a polymeric matrix (Flemming & Wingender, 2010). These consist of numerous components including microbial cells such as bacteria, archaea and protozoa (2-5%), water (major component that equals to about 97%), polysaccharides (1-2%), extracellular proteins (1-2%), DNA and RNA (1-2%), and ions (Takhistov & George, 2004). All components of the biofilm are embedded in a self-produced extracellular matrix that helps to protect microorganisms from harsh external forces.

Aquatic biofilm formation results from an initial attachment of a free microorganism to a surface. This inception is aided by the *Van der Waal* forces of attractions and hydrophobic effects (Kolter, 2015). Once the initial colony is fixed, the bacteria permanently anchor themselves using adhesion structure-like pili (Ryder & Mascarenhas, 2007). Increased hydrophobicity can also make the microorganisms fasten themselves together to enduringly form a colony. During attachment, the microorganisms secret an extracellular polysaccharide substances (EPS) that encloses the microorganisms, creating a biofilm, which thereafter grows in size due to the constant addition of microorganisms (Bonnineau, Tlili, Faggiano, Montuelle, & Guasch, 2013; Stoodley et al., 2002). Throughout this entire process, individual cells within the biofilms communicate with one another by means of quorum sensing. This allows for the microbial community to regulate gene expression, thus respond to environmental stimuli, new food sources, and coordinate the recruitment of other microorganisms (Dobretsov, Teplitski, & Paul, 2009). Once too large, the biofilm undergoes dispersion, in which the microorganisms spread to colonize other areas. This reduces competition for nutrients within the colony (Takhistov & George, 2004).

1.8.2. Retention of Cryptosporidium Oocysts in Aquatic Biofilms

Aquatic biofilms serve as reservoirs for *Cryptosporidium* oocysts, creating favourble microclimates for their survival and dispersion. Wolyniak DiCesare, Hargreaves, & Jellison (2012a) inoculated *C. parvum* oocysts in biofilms, then exposed them to solar radiation. The same solar intensity was simultaneously exposed to free-floating oocysts, and overtime there were about double the number of oocysts attached to the biofilm bases compared to oocysts suspended freely in water. The retention of oocysts in biofilms is facilitated by surface roughness

and thickness. Through qPCR and COMSTAT analyses, Koh, Clode, Monis and Thompson (2013) determined that the retention of *Cryptosporidium* oocysts significantly increases ($p< 0.05$) as biofilms increase in thickness. Other studies have shown a positive correlation between high standard deviation of biofilm thicknesses (indication of surface roughness) and oocysts attachment. These observations of biofilm roughness were suspected to be influenced by seasonal changes (Wolyniak DiCesare, Hargreaves, & Jellison, 2010; Wolyniak DiCesare, Hargreaves, & Jellison, 2012b). Furthermore, oocysts can remain intact to biofilms despite the disturbance of flowing water. For example, Searcy, Packman, Atwill, & Harter (2006) used a flow-cell system and nutrient enrich growth media to observe the capture and retention of *C. parvum* oocysts in *P. aeruginosa* biofilms. After 24 hours, the study found oocysts attached to biofilms (Figure 1.7.), which were released 1 hour later once the flow rate increased by 40-folds. Overall, substantial evidence indicates that biofilms recruit and retain *Cryptosporidium* oocysts, protecting them from deactivation via solar radiation and other environmental stressors.

Another benefit that biofilms potentially provide, is a suitable microclimate for oocysts development and reproduction. Within the past decade, there has been an increasing number of studies showing the *in vitro* extracellular multiplication of *Cryptosporidium* oocysts in both cell and cell-free cultures (Arrowood, 2002; Clode et al., 2015; Hijjawi, 2004, Hijjawi, Meloni, Ryan, Olson, & Thompson, 2002). For example, Hijjawi (2010) composed a review on various animal and human host cells that are capable of *Cryptosporidium* growth, including BFTE, MDCK, BS-C-1, HCT-8 and Caco-2. In addition, Hijjawi, Estcourt, Yang, Monis, & Ryan (2010) used a cell-free maintenance media to cultivate *C. hominis* and was able to visually observe oocysts' developmental stages without excystation. Finally, Koh et al. (2013) was the first to present significant evidence of extracellular growth of *C. parvum* oocysts in *P. aeruginosa* biofilms under aquatic conditions. The study found a significant increase in *C.*

parvum of 2–3 folds ($P < 0.001$) over a 6-day period as the biofilms matured. Also, the life-cycle stages of *C. parvum* were identified using confocal microscopy, which showed that the oocysts were multiplying at different life-cycle stages within the biofilms. Therefore, recent studies have demonstrated that *Cryptosporidium* oocysts can multiply and grow outside hosts. Although, evidence conveying oocysts multiplication within biofilms is relatively new, thus warrants further research.

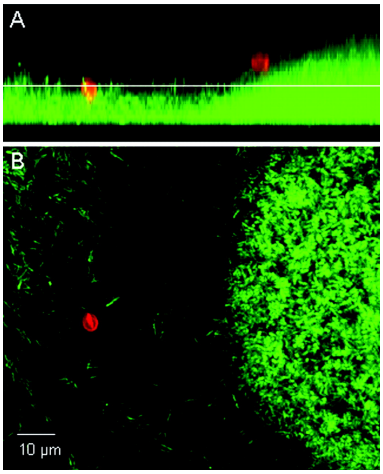

Figure 1.7.: The entrapment of *C. parvum* oocysts (red) in a *P. aeruginosa* biofilm (green). Captured under confocal microscopy using Cy3-conjugated monoclonal antibody to stain oocysts and green fluorescent protein to stain the biofilm. Magnification, 60X. Source: Searcy et al. (2006).

1.8.3. Biofilm Sampler and Water Monitoring

Biofilms serve as natural filters in water, hence their analysis could provide critical information about the microbial community that passes along streamlines. Much work has been completed on the recruitment, retention and transportation of pathogens in aquatic biofilms including *Cryptosporidium* investigations (Luo, Jedlicka, & Jellison, 2016; Wolyniak DiCesare, Hargreaves, & Jellison, 2009; Wolyniak et al., 2010; Wolyniak DiCesare et al., 2012b). However, very few studies have focused on using biofilms for water monitoring purposes.

The *biofilm sampler* is an apparatus used to monitor various species in water. The innovation permits biofilm development on removable surfaces, which are analyzed to determine microbial composition and behaviour. Original *biofilm samplers* were used in laboratories, where detachable studs were exposed to flowing water for 2-3-weeks, allowing for mature biofilms to form. Chemical manufacturers would then test these biofilms against various antimicrobial products such as biocides, antiseptics, drain cleaners and antibiotics (Caproco, 2003). Laboratory investigations have since adopted the *biofilm sampler* technique in flow cell systems, where a medium is continuously pumped through capillary tubing, promoting the attachment and growth of biofilms to a flow cell (glass prism), which is mounted under a microscope. Under these hydrodynamic conditions, the spatial distribution, composition and growth of biofilms can be assessed in real-time (Peterson et al., 2011).

Apart from laboratory investigations, *biofilm samplers* have a long history in drinking water distribution systems. Inspectors use the device to monitor biofilms in the interior walls of piping networks. The Robbins Device is one of the older and most popular forms of *biofilm samplers*. The device is placed in a section of a piping network, allowing for biofilms to form on removable coupons that fit in sampling ports. Robbins Devices are typically used to investigate biocidal activity on different surfaces. They are also used to determine the effects of velocity and turbidity on biofilm formation (Azeredo et al., 2016).

In terms of environmental assessment, *biofilm samplers* have been used to monitor various species in surface water. Fuchs, Katbeh-Bader, & Alkhateeb (2012) used a *biofilm sampler* to identify organisms in the Wadi Ar Rumman ravine, Jordan. The samplers were constructed using PVC pipes with glass inserts, providing a flat surface where the biofilms settled and grew (Figure 1.8a.). These biofilms were assessed to determine the impact of heavy metal pollutants on

species composition. Similarly, Tonetto, Peres and Branco (2012) used a *biofilm sampler*, which was constructed out of acrylic plaques and glass slides to monitor benthic communities in lotic waters of southern Brazil (Figure 1.8b.). Last, Peacock et al. (2004) used a *biofilm sampler* constructed out of Teflon tubing loaded with glass wool or Bio-Sep beads to monitor water-quality in a field bioremediation experiment in Tennessee (Figure 1.8c.). These biofilms were analyzed via PCR to determine microbial community structure.

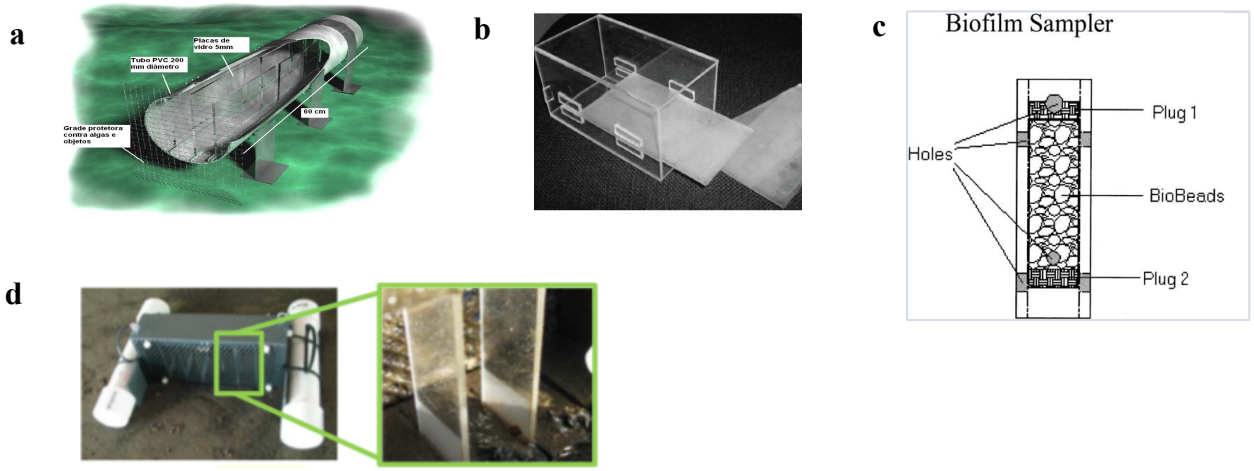

Figure 1.8.: Previous *biofilm samplers* used for surface water monitoring. (a) to assess the effects of heavy metal pollutants on species composition. (b) to investigate benthic communities in lotic waters (c) to monitor water-quality in a field bioremediation experiment. (d) to monitor the upstream and downstream prevalence of *Cryptosporidium* oocysts. Sources: Fuchs et al. (2012); Peacock et al. (2004); Philadelphia Water Department [PWD] (2014).; Tonetto et al. (2012).

1.8.4. Biofilm Sampler Technique for Detection and Molecular Characterization of Cryptosporidium spp. from Surface Water

Researchers at Lehigh University (Bethlehem, Pennsylvania) coined the "*biofilm sampler* technique" for *in situ* monitoring of *Cryptosporidium* in surface water. In collaboration with the Philadelphia Water Department (PWD), ongoing projects have monitored the upstream and

downstream prevalence of *Cryptosporidium* oocysts from various watersheds in Eastern Pennsylvania. The *biofilm samplers* were constructed out of metal-box frames containing glass microscope slides (Figure 1.8d.). After a 2-3-week period, the biofilms were analyzed for *Cryptosporidium* oocysts by a partial EPA Method 1623, which excluded the filtration step but abided by the IMS and fluorescent microscopy guidelines (Barnes-Pohjonen, 2012; McLeod, 2011; PWD, 2014).

Significant findings from these projects were compared to Method 1623's filtration to determine the difference between oocysts recovery rates. The *biofilm samplers* produced 46% positive (18/39), whereas EPA filters produced 43% positive (17/40) (Westerling, 2014). In addition, Barnes-Pohjonen (2012) found that Fluorescent *in situ* Hybridization (FISH) processing of biofilms attached to slides identified more positives than Method 1623, indicating that the biofilm/FISH method was more sensitive and had a lower detection limit. Furthermore, McLeod (2011) used the *biofilm sampler* technique to genotype *Cryptosporidium spp.* from biofilm samples. Through nested-PCR and 18S rRNA sequencing, multiple genotypes of *C. parvum* were identified in the Schuylkill River, and 75% were associated with human infections. Therefore, previous investigations have demonstrated that biofilm sampling is comparable, and sometimes even more sensitive than Method 1623's filtration. Moreover, it can be used to monitor *Cryptosporidium* species and genotypes, thus assert public health risk.

1.9. Summary

- *Cryptosporidium* is a protozoan parasite that causes cryptosporidiosis—a diarrheal disease that manifests through the oral-fecal route and can be life threatening to children and immunocompromised individuals.

- *Cryptosporidium* is a part of the Apicomplexa phylum. It was traditionally recognized as a coccidian; however, modern research suggests that it is now a gregarine. To date, there are at least 24 species/genotypes, and 65 subtype families of *Cryptosporidium.*
- *Cryptosporidium* was first described in 1907. Since then, it has been linked to sickness in livestock, particularly cattle. In 1971, *Cryptosporidium* gained public health interest after its first reported cases in humans.
- *Cryptosporidium* is a global burden. Developing countries are at greatest risk due to inaccessible technologies and healthcare infrastructure. In the Caribbean, *Cryptosporidium* has been detected in human populations and water; however, genomic diversity of species is low. There is also a gap in literature regarding *Cryptosporidium spp.* detection from environmental sources in the Caribbean.
- Global waterborne outbreaks of cryptosporidiosis have sporadically occurred, leading to major consequences. The 1993 Milwaukee outbreak remains the largest of its kind. Its aftermath prompted research, resulting in enhanced monitoring and detection methods including IFA-microscopy (Method 1623), PCR and gene sequencing.
- The sensitivity and specificity of *Cryptosporidium* PCR detection and gene sequencing are limited due to environmental inhibitors, false positives yielded from closely related species, and naturally low DNA contained in environmental samples.
- Alternative *Cryptosporidium* detection methods include: MAF and IFA microscopy, and ELISA antigen test. Although, these methods are also limited due to microscopic subjectivity and the cross-reactivity of stains/antibodies.
- Waterborne transmission is the primary source of human cryptosporidiosis. Various water parameters including pH, DO, DOC, hardness and temperature have shown to influence the survival and transport of *Cryptosporidium* oocysts. Indicator pathogens

including *E. coli* and fecal coliforms have shown to be both correlated and inconsistent with *Cryptosporidium* prevalence.

- Aquatic biofilms have also shown to improve the life expectancy of oocysts by providing suitable microclimates for protection, transportation and potential multiplication.
- Sampling biofilms via the *biofilm sampler* technique, followed by fluorescent microscopy, PCR and gene sequencing can be useful for monitoring *Cryptosporidium spp.* in surface water

Chapter 2: Introduction

2.1. Research Aim and Rationale

Global perspectives of cryptosporidiosis have evolved from that of a rare disease, to a fatal diarrheal disease that can be transmitted between humans, animals, food and water sources. Although surface water is recognized as the primary source of human cryptosporidiosis, regular surveillance in most countries is impractical due to high overhead costs. These expenses are associated with conventional filtration equipment, which have been standardized for use by the US EPA Method 1623. Notably, Method 1623 offers a universal approach that is internationally renowned for regulating *Cryptosporidium* from surface water. However, the technique only uses fluorescent microscopy, hence does not provide any information about *Cryptosporidium* at the species or genotype level.

Given the limitations of Method 1623, research presented in this dissertation focused on the development and evaluation of an alternative method referred to as the *biofilm sampler* technique for microscopic and PCR detection, and molecular characterization of *Cryptosporidium spp.* from biofilm samples. Based on the literature review, it is understood that biofilms recruit and entrap *Cryptosporidium* oocysts, thereby facilitate prolonged survival, dispersion and potential multiplication. Therefore, the development of the *biofilm sampler* approach could aid in the advancement of monitoring and source tracking practices.

It is important to note that the purpose of developing the *biofilm sampler* technique was not to replace conventional filtration methods; but rather offer an alternative method that could work in conjunction with multiple techniques applied to a set of aquatic sources that are well-known reservoirs of *Cryptosporidium spp*. Such sources may include biofilm, shellfish, benthic

macroinvertebrates, which have previously demonstrated to naturally accumulate *Cryptosporidium* oocysts (Reboredo-Fernández, Prado-Merini, García-Bernadal, Gómez-Couso, & Ares-Mazás, 2014; Staggs et al., 2015; Wolyniak et al., 2010).

In addition to biofilm sampling, this dissertation aimed to employ a variety of techniques including MAF microscopy, ELISA antigen testing, PCR and gene sequencing to detect and characterize *Cryptosporidium spp.* from cattle feces collected within the Black River watershed. The agreement between MAF and ELISA detections was also statistically evaluated. Records were maintained per sample (i.e., age, production type, diarrheal symptoms and farm management) for further interpretation of results. Several studies agree that cattle are major reservoirs of *Cryptosporidium spp.* that cause zoonosis. Thus, research in the discipline is critical for both human and animal health. It provides informative data, especially in the event of outbreak situations, and may aid in the development of strategies for limiting incidences of cryptosporidiosis throughout the watershed or alike environments.

Finally, the selected region of study was the Black River watershed of Jamaica because most studies in the Caribbean have focused on *Cryptosporidium* detection from human populations. Very few studies have investigated the population structure of different species and genotypes from environmental sources, including surface waters and animal feces. Specifically, in Jamaica, there is no available research on *Cryptosporidium* detection from environmental sources. This not only highlights the gap in research related to waterborne and zoonotic transmission, but also the difficulty in detecting and characterizing *Cryptosporidium spp.* from environmental samples. Based on these observations, further research is necessary to establish a database that provides critical information about existing species and genotypes that are geographically distinct and have the potential to cause zoonosis in the region.

2.2. Specific Objectives

2.2.1. Objective #1: Method development and evaluation of the biofilm sampler technique for Cryptosporidium oocysts entrapment and recovery efficiency from biofilm suspensions

Method development and evaluation of the *biofilm sampler* technique is important because it is a cost-effective and practical alternative to conventional filtration. As previously stated, EPA's Method 1623 is the universal method used for *Cryptosporidium* oocysts monitoring in surface water; however, the technique requires expensive filters that can only monitor oocysts at a specific location, at a single point in time. The *biofilm sampler* technique on the other hand, has proven to be a cost effective and reusable alternative that can monitor *Cryptosporidium* oocysts in surface water over a 2-3-week period. The following objective therefore, aimed to design a *biofilm sampler* that could optimize the growth of biofilms on polycarbonate substrate. The growth surface was hypothesized to produce thicker and rougher biofilms, thereby enhance the entrapment of *Cryptosporidium* oocysts. Thus, through confocal microscopy and COMSTAT2 analysis, biofilm thickness and roughness were measured and compared to previous studies in relation to biofilm retention of *Cryptosporidium* oocysts. The objective also aimed to evaluate the recovery efficiency of oocysts seeded into the same biofilms using the IMS-IFA technique and gridded coverslips. All results were statistically evaluated by calculating relative standard deviation (RSD) and are presented in section 4.1 of this submission.

2.2.2. Objective #2: Development of PCR assays to optimize Cryptosporidium detection and genotyping from environmental samples using selected primers

It is widely accepted that PCR assays for *Cryptosporidium* detection and genotyping from environmental samples are challenging to execute. This is due to low quantities of DNA, as well

as high prevalence of inhibitors and other microorganisms that interfere with the PCR reaction. This objective therefore aimed to use spectrophotometric analyses and temperature gradient tests to enhance PCR thermocycler conditions, using common CPB-DIAG (Johnson et al., 1995), KLJ (Jellison, Hemond, & Schauer, 2002), and XIAO nested primers (Xiao et al., 1999). Results from these analyses are presented in section 4.2. of this dissertation, where spectrophotometry was performed to determine adequate primer dilution, as well as the concentration of *Cryptosporidium* DNA present in each sample type. In addition, temperature gradient tests were performed to determine optimal annealing temperature, which generated the most distinct bands with minimal non-specific binding.

2.2.3. Objective #3: Detection and molecular characterization of Cryptosporidium spp. from biofilms in the Black River and tributaries

The aim of this objective was to use the *biofilm sampler* technique and PCR methods developed in Objective #1 and #2 to detect and characterize *Cryptosporidium spp.* from biofilms collected from 5 sites in the Black River and tributaries. The main purposes were as follows: 1) to detect *Cryptosporidium* oocysts via IFA microscopy and differentiate positive detections that have DAPI-visible nuclei; 2) confirm positive detections using a nested-PCR assay; 3) sequence the 18S rRNA and gp60 genes of PCR products and perform phylogenetic analysis to identify *Cryptosporidium spp.* and potential subtypes that contaminate the water source and thus, pose risk to public health. Results from these analyses are presented in section 4.3. of this submission.

2.2.4. Objective #4: Detection and molecular characterization of Cryptosporidium spp. from cattle feces in the Black River watershed

This objective aimed to detect and characterize *Cryptosporidium spp.* from 119 cattle fecal specimens collected from 10 farms within the Black River watershed. This involved the application of MAF microscopy, ELISA, PCR and gene sequencing techniques. The proportion of positives detected per method was correlated with variances between dairy and beef cattle. Gene sequencing and phylogenetic analysis was performed to identify *Cryptosporidium spp.* and subtypes, as well as to evaluate public health risk based on identified isolates, previously implicated in waterborne and zoonotic infections. Results from these analyses are presented in section 4.4. of this submission.

2.2.5. Objective #5: Evaluation of diagnostic agreement between MAF microscopy and ELISA antigen test for the detection of Cryptosporidium from cattle feces

Since all techniques have sensitivity and specificity limitations, as highlighted in section 1.6., multiple methods were employed to verify positive detections from cattle feces. This objective therefore, aimed to assess the diagnostic (inter-rater statistical) agreement between MAF microscopy and ELISA, by calculating Cohen's kappa coefficient (*k*). The *k*-values were interpreted based on a well-established classification scheme. This form of statistical analysis took into account the possibility of agreement by chance. PCR was not included in the analysis due to low sensitivity in cattle feces, compared to MAF and ELISA techniques. Results are presented in section 4.4. of this submission.

2.3. Summary of Experimental Workflow

As described in Objective #s 3-5, multiple techniques were employed to detect and genotype *Cryptosporidium spp.* Figure 2.1. provides a summary of experimental workflow.

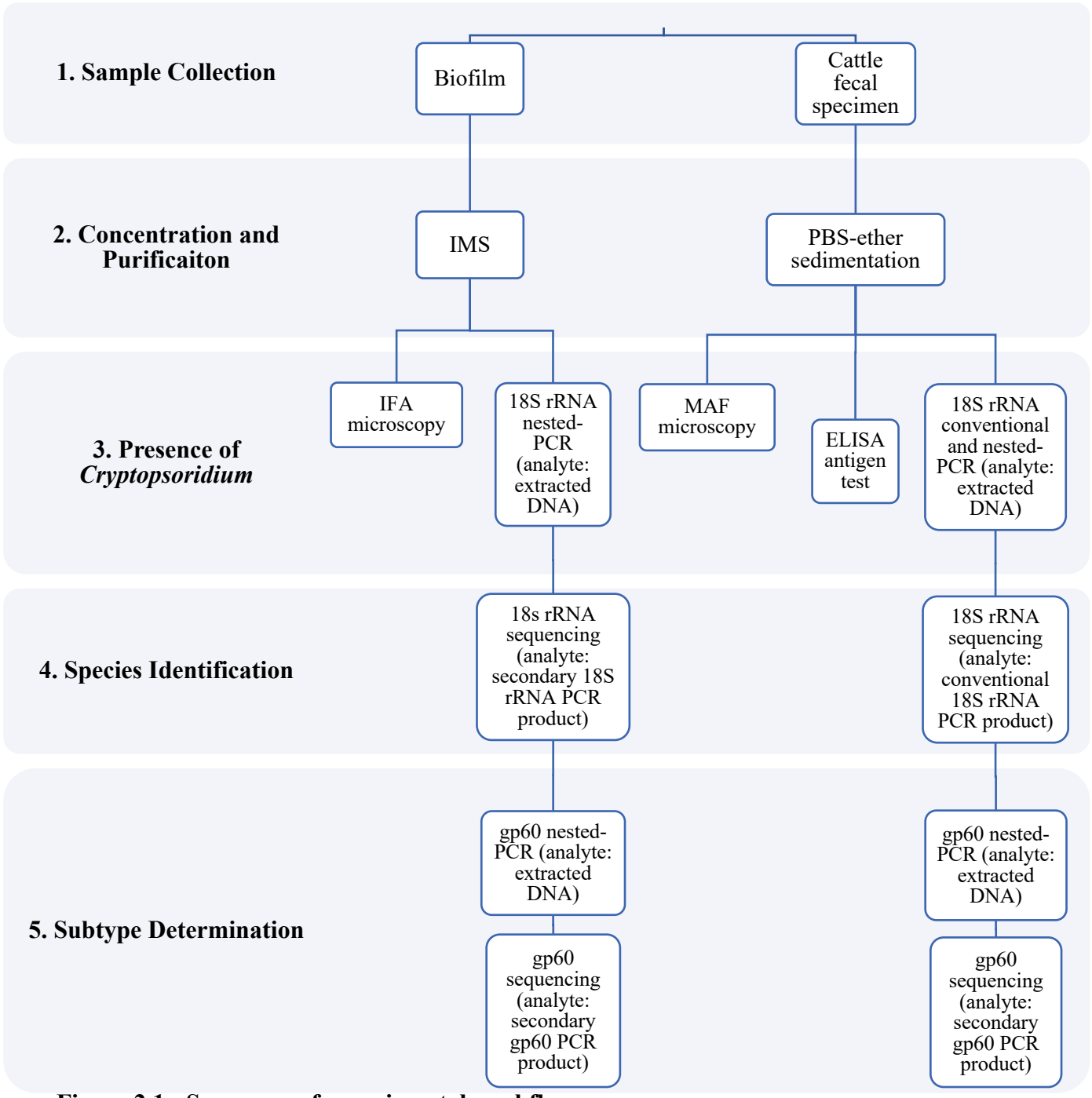

Figure 2.1.: Summary of experimental workflow.

2.4. General Description of Study Region

In Jamaica, there are 14 parishes and 26 watersheds. The Black River watershed is named after the dark hue of its mainstream river bed, formed after years of decomposing vegetation. The watershed is predominately situated in St. Elizabeth—a rural parish located in the southwestern region of the island. St. Elizabeth is 1210.7 km^2 and had a population of 150,993 in 2012 (Statistical Institute of Jamaica, 2017). The watershed also encompasses the southern regions of Westmorland, St. James and Trelawny, as well as the northwestern region of Manchester (Figure 2.2). The river itself measures 54.4 km and is maneuverable for about 40 km. The river network is supported by many tributaries including YS, Broad, Grass and Horse Savannah, which forms the longest river system in Jamaica (Fiwi Roots, 2016).

Figure 2.2. Fourteen parishes and twenty-six watersheds of Jamaica. Multi-colour basemap displays 14 parishes. This is overlaid by the solid black-lined map, which displays 26 watersheds. Adapted and modified from sources: Holness (2016) and NEPA (2003).

The river network is categorized into two main constituents; the Upper Morass and Lower

Morass (Figure 2.3). The Upper Morass includes the highest point and middle region of the river, flowing through a swampy section, which serves as a settling basin and sink for nutrients (Caribbean Birding Trail, 2015; Mordab, Hong Yuan, & Hu, 2013). The Lower Morass is Jamaica's largest wetland area—a declared wetland of importance under the United Nation's international treaty for the conservation and sustainable use of wetlands (Smith Warner International, 2005). From the Lower Morass, the river empties into the Caribbean Sea.

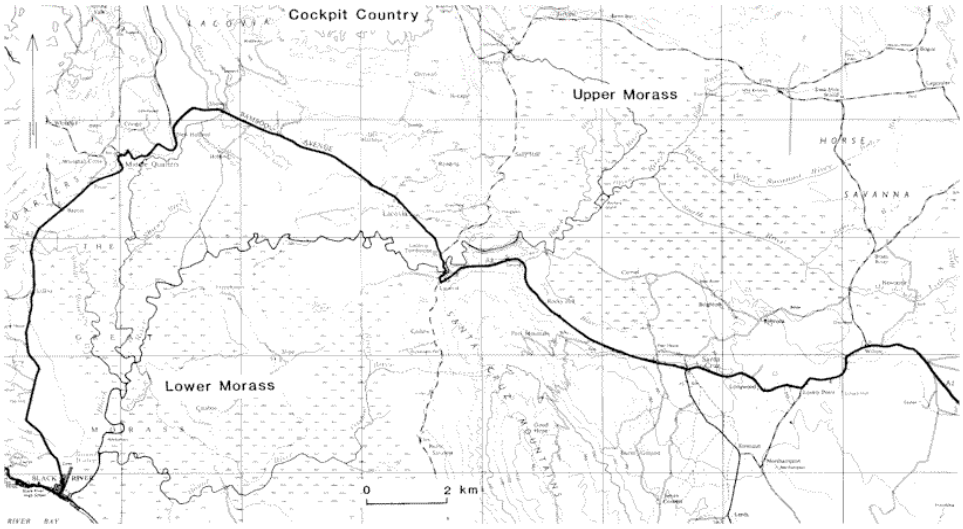

Figure 2.3.: Upper and Lower Morass of the Black River watershed. Source: Björk (2013).

The Black River receives influences from many human activities. Currently, the river is used for irrigation, fish and shrimp harvesting, and to take tourists on motor boat excursions. According to the Jamaican Protected Areas Trust Limited [JPAT] (2016), there is an urgent need for protection of the Black River banks due to increasing threats, including agricultural pollution. The river occupies the entire St. Elizabeth parish, which is known as the "bread basket" of the island, producing 22% of the nation's food (Jamaica Observer, 2014). Thus, agricultural production such as, livestock rearing, farming, irrigation and fertilization/cultivation of crops, collectively impact the water quality of the river.

Chapter 3: Materials and Methods

3.1. Pilot Study: Biofilm Development and Oocyst Recovery

3.1.1. Biofilm Sampler Design

Three customized *biofilm samplers* were created using a metal frame measuring 21 cm x 4 cm x 14.8 cm. Each frame had a front and back surface with permeable holes, allowing for water to infiltrate, while restricting the flow of large organisms, organic material and debris. At the base of the apparatus, there were four holes, which enabled the use of long pegs for nailing the sampler into the ground opposed to a conventional weight system as shown in section 1.8.3. (Figure 1.8d.). This feature secured the apparatus in an upright position despite high stream velocity. Inside the metal frame enclosed 8 slots for removable slides measuring 3.5 cm x 0.24 cm x 14 cm, which were made out of polycarbonate (plastic) substrate. Figure 3.1. provides photographs and illustrates the configuration of the *biofilm sampler* apparatus.

The rationale for using plastic slides rather than previous glass surfaces was to optimize biofilm development. Microorganisms attach more readily to hydrophobic nonpolar surfaces such as plastics compared to hydrophilic materials such as glass and metals (Bendinger, Rijnaarts, Altendorf, & Zehnder, 1993; Fletcher & Loeb, 1979; Pringle & Fletcher, 1983). In addition, biofilms develop better on rough surfaces compared to smooth surfaces because they obstruct shear forces and produce a greater surface area for microbial colonisation (Charackalis, 1990). Last, the slides were positioned parallel to water flow to reduce collision and attachment of large organisms and debris. The positioning of the slides also facilitated the attachment of adhesive microorganisms forming EPS layered biofilms. Refer to, Figure G.I. in Appendix G for photographs of the *biofilm sampler* apparatus.

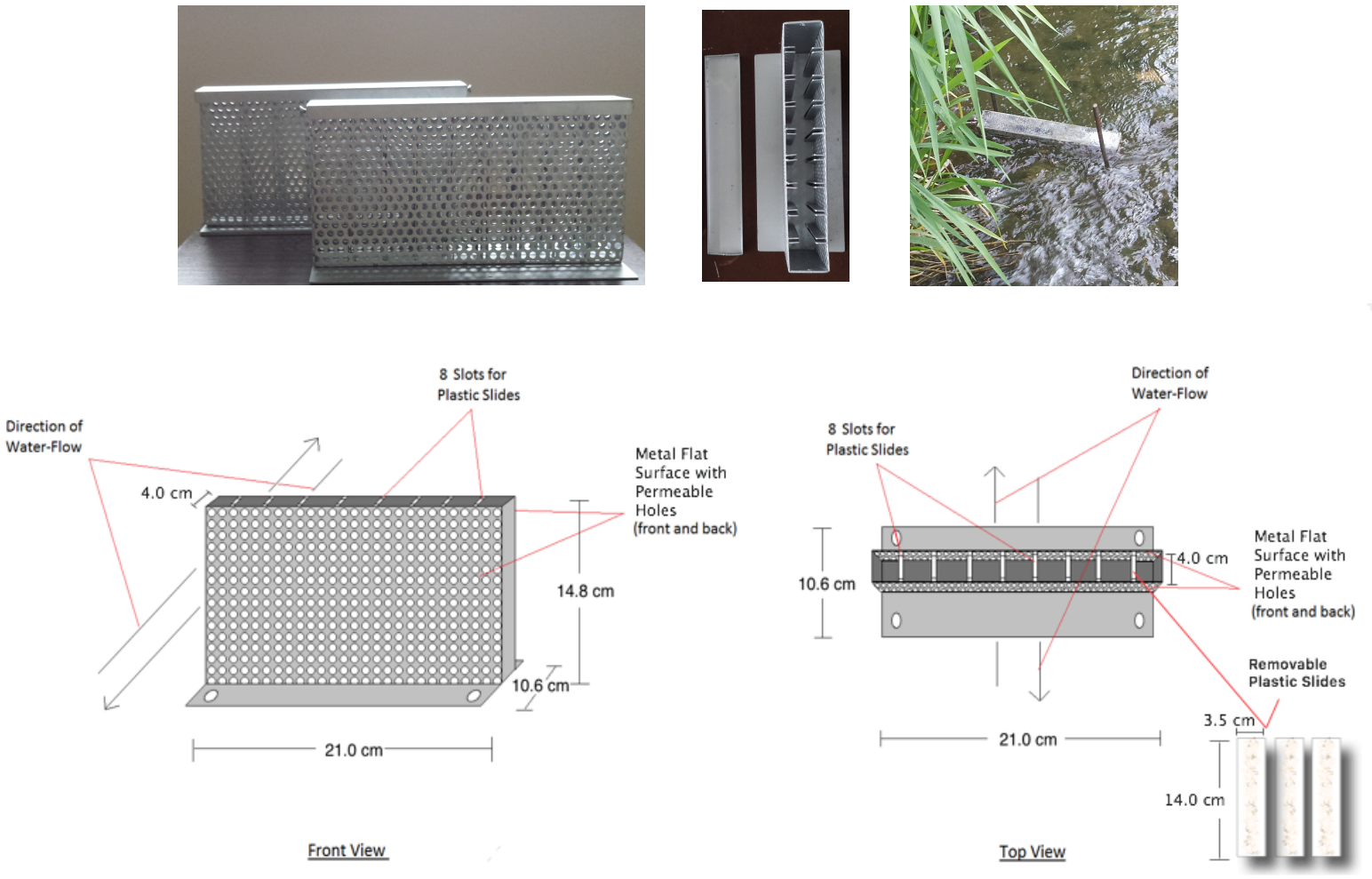

Figure 3.1.: Photographs and Diagram of the *biofilm sampler* apparatus with removable slides made out of polycarbonate material. Shown from the front and top view, illustrating the framework, dimensions and direction of water flow.

3.1.2. Sample Collection

During the spring of 2016, the *biofilm sampler* was inserted into shallow waters for 3-weeks, at approximately 50 cm depth in Alder Creek (Petersburg, Ontario). The specific timeframe was selected based on previous investigations, which found that mature biofilms require somewhere between 2 and 4-weeks to form complex structures, which generally plateau after the second

week (Hunt & Parry, 1998). Moreover, longer incubation periods would have resulted in sloughing; the sporadic detachment of large portions of the biofilm from its main structure (Kaplan, 2010).

A total of 4 plastic slides were introduced into the *biofilm sampler*, which served as growth surfaces for biofilms to develop on overtime. A glass reference-slide was also incubated alongside the polycarbonate substrate for comparative analysis. Prior to submergence in water, the slides were rinsed with 100% ethanol to remove any surface dirt or contamination. After 20 days, the slides were removed and put in a moist chamber, lined with a damp cloth to avoid dehydration. All samples were transported to the lab in an ice cooler, then stored in a refrigerator maintained at 4°C for analysis within 48-hours.

3.1.3. Fluorescent Staining and Confocal Z-stack Imaging of Biofilms

One half of each biofilm-slide (n=4) was stained with either Streptavidin Texas Red™ Conjugates or SYTO™ 9 or Green fluorescent stains SYTO™ 9 Green Fluorescent Nucleic Acid (Table A.III., Appendix A). First, a 1:100 dilution was made from the stains, which was loaded onto the biofilms and incubated for 45 minutes at 37° C. Second, a Nikon D-Eclipse C1 si confocal microscope with EZ-C1 3.80 software was used to capture layered images of the biofilms from base to top using the z-stack icon at 40X objective. Each z-stack contained layered images of 2 µm increments going down through the biofilms (Koh et al., 2013). Therefore, the number of z-stacks per biofilm varied according to thickness.

The red or green fluorescence of the biofilms was excited with 632 nm and 543 nm lasers, respectively. A total of 8 separate z-stack images were acquired for each biofilm slide from a

single channel, which met criteria outline by Heydorn et al. (2000). Scan locations were randomly selected in the central region of the slides to exclude disrupted surfaces or excessive biofilm accumulation formed after removing the slides from the *biofilm sampler* slots. Last, the confocal images produced in EZ-C1 3.80 were saved as .ics files, then downloaded into the NIS-Elements AR 3.10 imaging software. Three-dimensional images were created in ND Viewer (multidimensional image display) using the Volume Projection icon. Scale bar was added to all images.

3.1.4. COMSTAT2 Analyses of Biofilms Thickness and Roughness

Individual z-stack images of the biofilms were exported as .ics files from NIS-Elements AR 3.10 and downloaded into Microsoft Windows ImageJ Java 1.8 software (open source provided by the National Institute of Health [NIH]). These images were converted into greyscale and ome-tiff format. The COMSTAT2 function was selected and the biofilms were analyzed with Automatic Threshold (Otsu's method) and Connected Volume Filtering (CVF). The following parameters were assessed:

1. average thicknesses: the highest points (μm) of all pixel-columns from each z-stack layer (excluding holes and cavities) from which the mean value is calculated (Vorregaard, 2008).
2. maximum average thickness: the highest pixel-column (μm) from each z-stack layer, from which the mean value is calculated (Vorregaard, 2008).
3. Dimensionless roughness coefficient (R_a*): average deviation from the average thickness (normalized and dimensionless).

The following is the computing definition of dimensional roughness coefficient, according to Comstat 2 (2018):

$$R_a^* = \frac{1}{N \cdot t_{avg}} \sum_{i=1}^{slices} spots[i] \cdot (thickness[i] - t_{avg}) \quad (1)$$

where N is the total number of spots found, *spots* [i] is the spots of slice i, *thickness* [i] is the thickness defined by slice i, and t_{avg} is the average thickness found in the thickness distribution. Biofilms with low R_a* were considered to be more homologous, thus smoother than biofilms with high R_a*.

3.1.5. Calculating IMS-recovery Efficiency of Non-viable C. parvum Oocysts Seeded into Biofilm Suspensions

The remaining halves (*n*=4) of the biofilms were suspended in water then seeded with non-viable *C. parvum* oocysts to calculate IMS-recovery efficiency. Roughly, 19.5 cm^2 of intact biofilm (area of a standard microscope slide) was scraped from each slide using a sterile scalpel and suspended into a cuvette contain 12 ml of filtered creek water. The water was filtered through a 0.2 µm Pall Membrane Filter (Sigma-Aldrich, Inc., St. Louis, MO) as in Wolyniak et al. (2010), to maintain physical conditions for the natural microbial assemblages prior to IMS. These suspensions were transported to the lab, then seeded with 50 µl of non-viable *C. parvum* oocysts from the Crypt-a-Glo™ G/C Direct Comprehensive Kit (Table A.I., Appendix A). This was approximately 10,000 oocysts per suspension.

Turbidity was measured using an Orion™ AQ4500 Turbidimeter (Thermo Fisher Scientific Inc., Waltham, MA). Then, the mixtures were centrifuged at 1500 X g for 10 minutes. The supernatants were poured off and the pellets were subsequently recovered via IMS, as described in section 3.2.2. The only difference was that the final IMS products were suspended into 200 µl of 1.0 N NaOH.

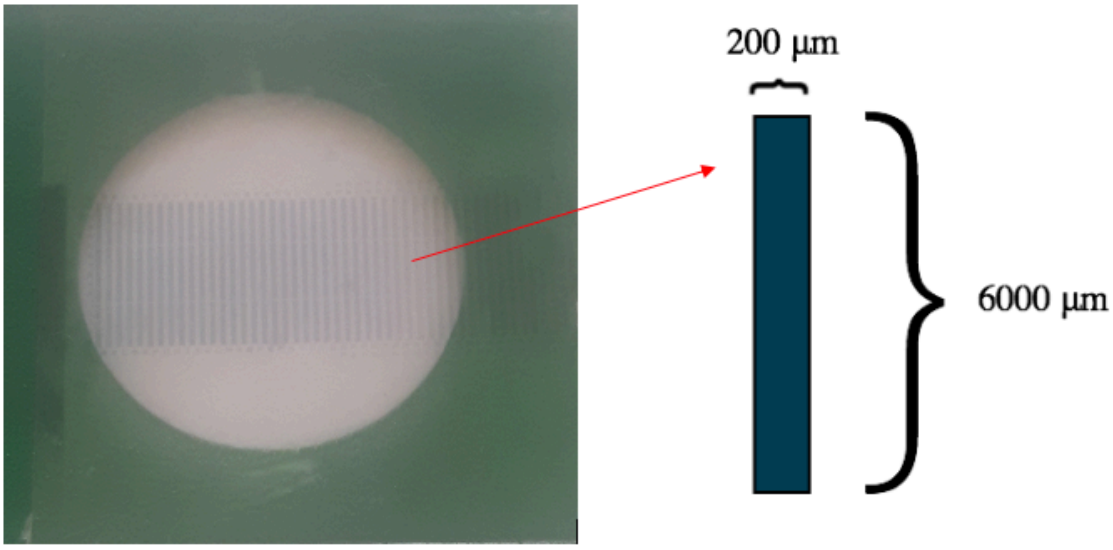

Figure 3.2.: REF coverslip mounted onto a well slide to facilitate quantification of *Cryptosporidium* oocysts. A total of 35 columns within the boundary of the well. Arrow pointing to diagram showing the dimension of a single column.

Fifty microlitres of IMS products were transferred to 15mm-SuperStick™ well slides (*n*=4). The slides were dried and stained with Crypt-a-Glo™ as described in section 3.2.3. Relocatable-grid reference (REF) coverslips (Pang, 1996) were mounted onto the slides and the number of oocysts were quantified going down individual columns (Figure 3.2.). REF coverslips were implemented, rather than traditional clear coverslips in order to facilitate quantification. Previous studies have adapted REFs for asbestos fiber-counting by microscopists, which have

demonstrated to improve accuracy, as well as provide opportunity for re-examination (Harper, Slaven, & Pang, 2009; Pang & Harper, 2008).

In total, there were 47 columns of examination according to the coverslip, 35 of which were fully within the boundary of the well. The dimension of each column is 6000 µm x 200 µm. By counting total N oocysts in Z number of columns, the concentration of oocysts was calculated as follows:

$$\text{Conc. of oocysts} = N \times \frac{\text{Area of the well}}{Z \times \text{area of one column} \times \text{droplet volume} \times \text{dilution factor}} \quad (2)$$

Note: 50 µl fills the well completely

3.2. IFA Detection of *Cryptosporidium* Oocysts from Biofilm Suspensions

3.2.1. Sample Collection

Five *biofilm samplers*—each containing 8 plastic slides—were inserted into shallow waters in the Black River network. In July 2016, two samplers were inserted at Site A and Site B; while in February 2017, three samplers were inserted at Site C, Site D and Site E (Figure 3.3.). The samplers remained submerged under water for a period of 20-22 days. Once the samplers were recovered, the slides were carefully removed without disturbing the intact biofilms. A sterile scalpel was used to scrape off the biofilms from the slides (front and back) into a Whirl-pak® bag containing 40 ml of filtered site water, as previously described in section 3.1.5. Each bag contained biofilms scraped from 4 slides. The samples were immediately transported to the

University of the West Indies (UWI), Microbiology lab in an ice cooler and stored in a refrigerator maintained at 4°C.

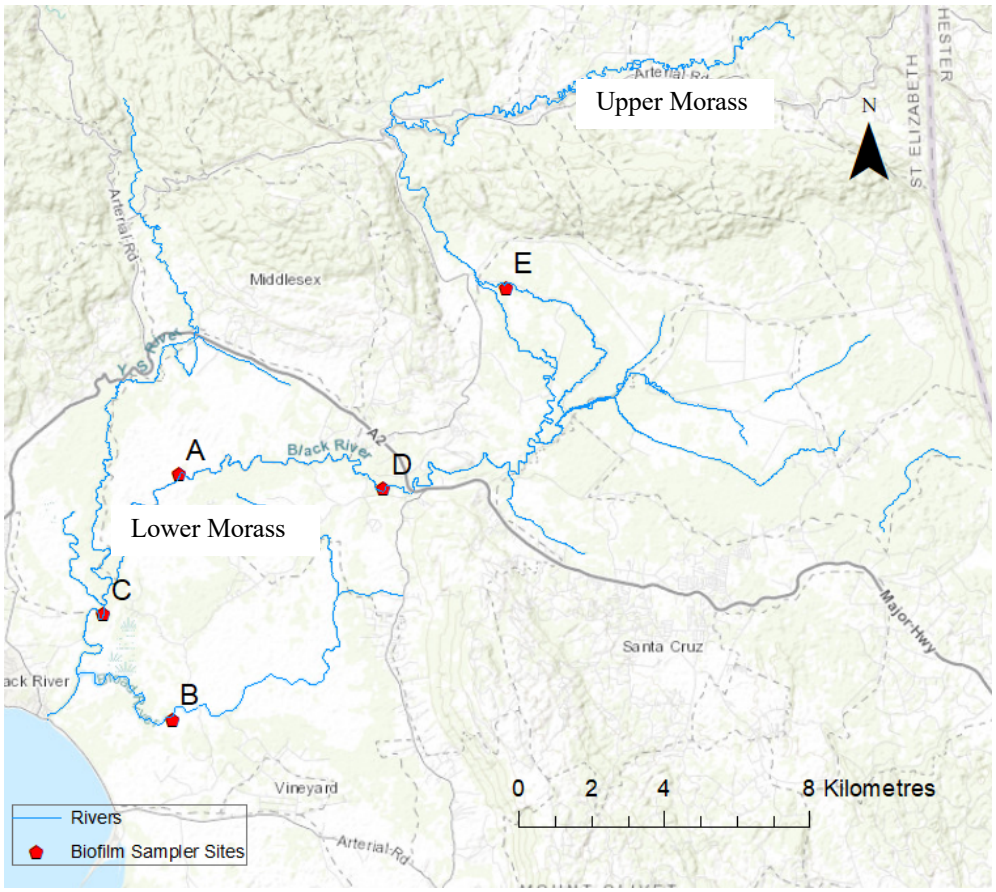

Figure 3.3. *Biofilm sampler* Sites A-E in the Black River Network, St. Elizabeth, Jamaica. Solid grey lines indicate major highways and roads; dashed lines indicate regional boundaries. Map created in ARCMap10 (ESRI Canada Limited).

3.2.2. Centrifugation and IMS

Centrifugation and IMS was performed within 48 hours of sample collection. The objective was to first concentrate biomass, then purify any oocysts contained within the biofilm suspensions. The Dynabeads™ anti-*Cryptosporidium* kit (Table A.I., Appendix A) was used to perform IMS

as recommended by EPA Method 1623 (US EPA, 2005). The procedure uses magnetic beads that are coated with monoclonal antibodies designed to bind specifically to *Cryptosporidium* oocysts. All steps followed the manufacturer's protocol, except for minor adjustments discussed below.

First, 40 ml of biofilm suspension was divided into 4 centrifuge tubes (10 ml ea.). The tubes were centrifuged at 1500 x g for 10 minutes allowing for biomass to pellet, and the supernatant was carefully discarded. When necessary, the pellets were adjusted to 500 µl or less by splitting the pellet into halves using a disposable applicator stick, followed by re-centrifugation. Each pellet was added to a L-10 tube containing 2 ml of SL™ Buffers. Subsequently, 100 µl of Dynabeads™ was added to the mixture. A positive control consisting of approximately 1.0 X 10^3 of non-viable *C. parvum* oocysts was processed alongside the test samples.

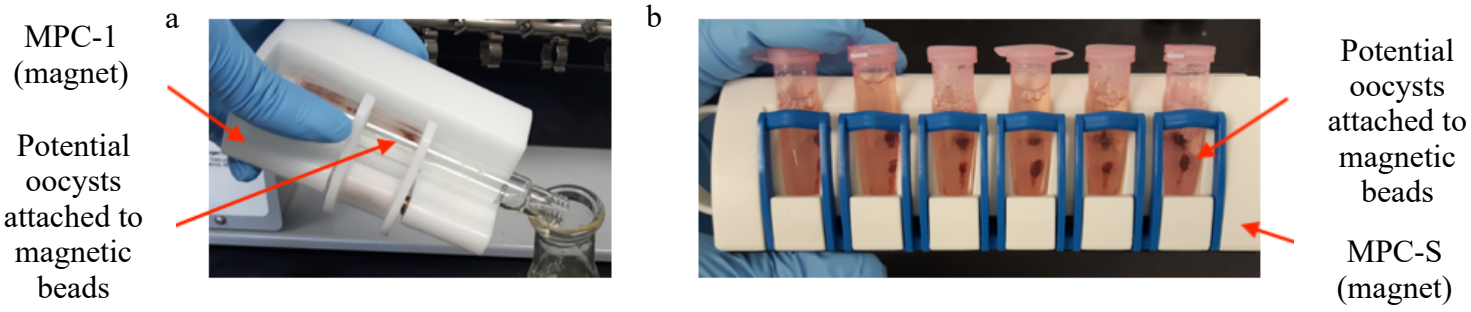

Figure 3.4.: MPC™ magnets used for oocysts purification. L-10 tube attached to MPC-1 magnet (a) while supernatant is poured off. Microcentrifuge tubes in MPC-S magnet (b).

Here, a pH adjustment step was added to the protocol. The pH levels from all L-10 tubes were measured using an Orion™ 9810BN Micro pH Electrode (Thermo Fisher Scientific Inc., Waltham, MA). While the electrode was emerged in the solution for approximately 2 minutes, pH was adjusted to 7 by adding either 1.0 N NaOH or 0.1 N HCl, depending on whether the

solution was too acidic or basic. To avoid cross-contamination, the electrode was rinsed thoroughly with distilled water and dried between measurements, as recommended in the manufacturer manual. In a previous study, Khun, Rock and Oshima (2002) made these adjustments and determined that a neutral pH level improved oocysts recovery by 26.4% compared to unadjusted samples.

The L-10 tubes were inserted into a rotating mixer and incubated for 1-hour at 18-20 rpm. After incubation, the tubes were removed and attached to a Magnetic Particle Concentrator (MPC)^{TM}-1. The beads were captured on the flat side of the tubes after rocking by hand for 2 minutes. While attached to the magnet, the caps were removed, and the supernatant was poured off (Figure 3.4a.). The magnet was detached, and the beads were resuspended into 400 µl of 1X SL^{TM} Buffer A, then transferred into corresponding 1.5 ml microcentrifuge tubes. Each microcentrifuge tube was placed in a second magnet, the MPC^{TM}-S and rocked by hand for 1 minute (Figure 3.4b.). The supernatant was removed leaving potential oocysts bound to the beads, which were resuspended in 50 µl of 1.0 N NaOH.

While the manufacturer's protocol recommends a final dissociation step, this was not performed. Normally, dissociation involves adding 5 µl of 0.1 HCL to the solution to detach oocysts from the magnetic beads. However, due to reports of poor dissociation, studies perform direct analyses to oocysts bound to the beads (Jiang et al., 2005; Xiao, Alderisio, & Jiang, 2006). Thus, 5 µl of each product was transferred to a Spot-On^{TM} well slide and air dried in preparation for IFA staining. The remaining products were stored in a freezer maintained at -20 °C until DNA extraction.

3.2.3. IFA Microscopy

IFA microscopy was performed to examine biofilms using the Crypt-a-Glo™ Comprehensive Kit (Table A.I., Appendix A) as recommended by the EPA Method 1623 (US EPA, 2005). A total of 78 replicates were examined from 24 biofilm suspensions collected from 5 sites. First, the IMS products previously transferred to Spot-On™ slides were air-dried and fixed with methanol. Fifty microlitres of DAPI stain was added to each well and incubated at room temperature for 1 minute. The slides were rinsed with SureRinse™ wash buffer. Once dried, one drop of Crypt-a-Glo™ antibody reagent was added to each well. The slides were incubated for 45 minutes at 37° C, then rinsed with the wash buffer. To reduce non-specific background fluorescence, one drop of BlockOut™ counterstain was added and incubated for 1 minute at room temperature. The slides were rinsed and a drop of No-Fade™ mounting medium was added along with coverslips.

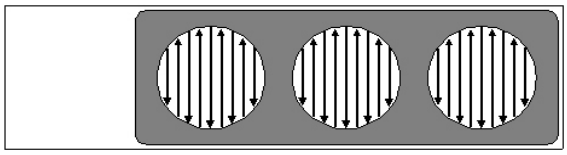

Figure 3.5.: Method 1623 examination pattern of well slide containing IFA-stained *Cryptosporidium* Oocysts. Protocol recommends that each well should be scanned in a systematic fashion (i.e., up-and-down pattern). Source: US EPA (2005).

A Nikon D-Eclipse C1 si confocal microscope with EZ-C1 3.80 software was used to identify *Cryptosporidium* oocysts. Green fluorescence was excited using a 543 nm laser. Figure 3.5 shows the examination pattern for all slides. The examination process started with 40X magnification objective to identify the presence of oocysts followed by confirmation with 100X oil immersion objective. The criteria for positive detection were: 1) brilliant apple green fluorescence; 2) round or oval shape; and 3) approximate diameter of 4-8 μm, confirmed with scale bar. In addition, DIC objective was used to identify internal morphological characteristics,

while a DAPI filter was used to identify sporozoites, which appeared a blue colour. The positive control described in section 3.2.2. was examined alongside the samples.

3.3. MAF Detection of *Cryptosporidium* Oocysts from Cattle Feces

3.3.1. Sample Collection

One hundred and nineteen cattle fecal samples were collected from 10 farms located in the Black River watershed during February, March and October 2017. Only 1 specimen was collected per animal. Figure 3.6. displays the geographic locations of each farm and the number of samples collected to scale. Specimens were transported to the microbiology laboratory in an ice cooler and stored in a refrigerator maintained at -20°C until further processing.

Fifty-nine of the specimens derived from beef cattle and 60 were from dairy cattle. Qualitative data pertaining to age, sex, production type, and diarrheal symptoms were recorded at the time of sample collection for further interpretation of results. The age of cattle ranged between 0.1 and 11 years.

Selection criteria of farms included: small to medium family owned dairy or beef herds, ranging from 20 to 100 cows per farm. Also, each cow was either born or resided on the farms for at least 4 weeks. Additional information about drinking water sources(s), land rearing/flooring system, hygiene maintenance, and cow-calf separation was recorded at the time of sample collection and during follow up phone interviews, to obtain a general consensus of farm management.

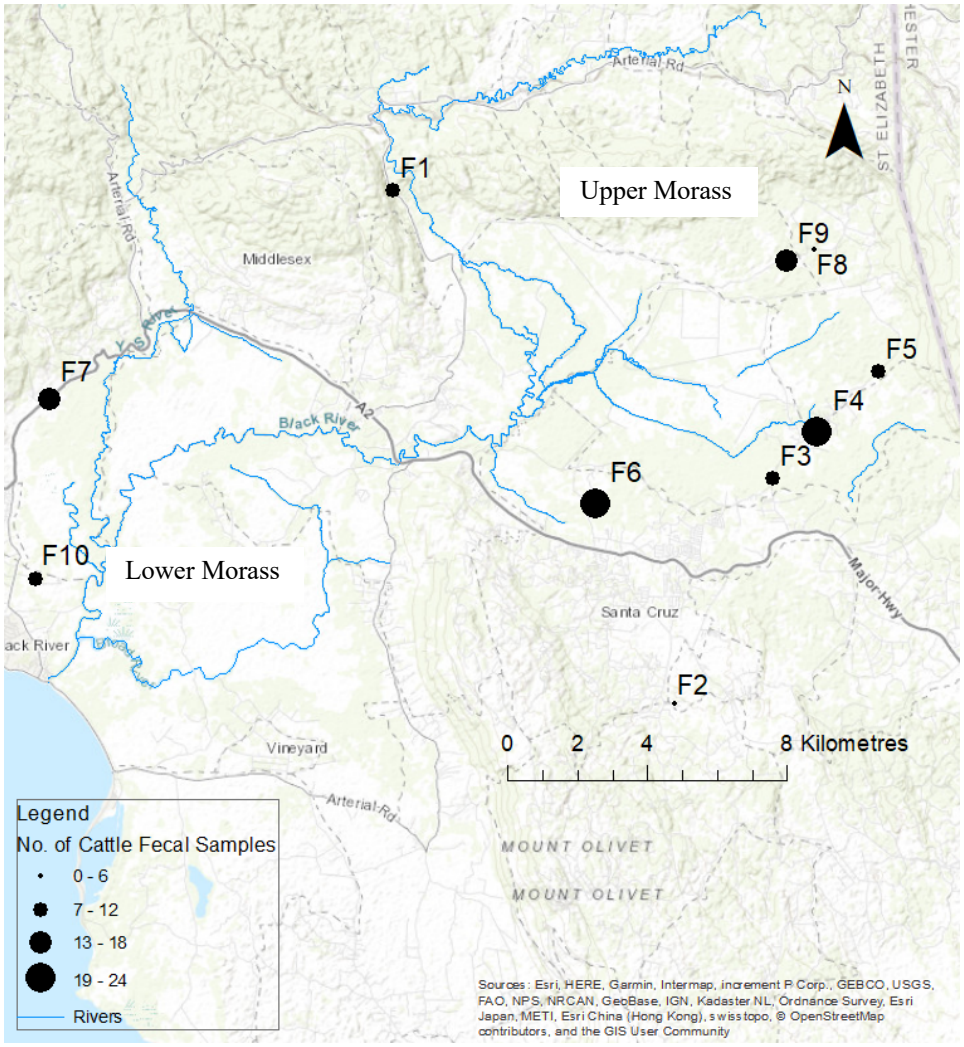

Figure 3.6.: Cattle farms F1-F10 and distribution of fecal specimens collected in the Black River watershed, St. Elizbeth, Jamaica. Solid grey lines indicate major highways and roads; dashed lines indicate regional boundaries. Map created in ARCMap10 (ESRI Canada Limited). A total of 60 and 59 specimens were collected from individual dairy and beef cows, respectively.

3.3.2. Filtration and Phosphate Buffer Saline (PBS)-Ether Sedimentation

One of the major difficulties in recovering *Cryptosporidium* oocysts from fecal specimens is attaining the required concentration for microscopy and PCR. In light of this challenge, filtration and PBS-ether sedimentation was used to concentrate oocysts from cattle feces. This procedure typically uses 10% formalin rather than PBS; however, formalin is a known inhibitor of PCR

assays (Groelz et al., 2013). According to Dietrich et al. (2013), formalin causes random fragmentation of short DNA debris, which bind to the polymerase enzyme, thus interferes with the reaction. In addition, PBS is a better alternative because it is non-toxic, which prevents the rupturing and shrivelling of cells due to osmosis (Martin et al., 2006). Given these factors, several studies have adopted PBS-ether sedimentation prior to microscopic and PCR detection of *Cryptosporidium* oocysts (Freire-Santos et al. 2002; Gómez-Couso, Méndez-Hermida, & Ares-Mazás, 2006; Mendonça et al., 2007).

In the following research, PBS-ether sedimentation was performed on 119 cattle feces as per Waldman, Tzipori, & Forsyth (1986) with minor adjustments. A positive control sample seeded with approximately 1.0 x 10^3 non-viable *C. parvum* oocysts was assessed alongside the samples. Table A.II. in Appendix A provides further details about all reagents used for sedimentation.

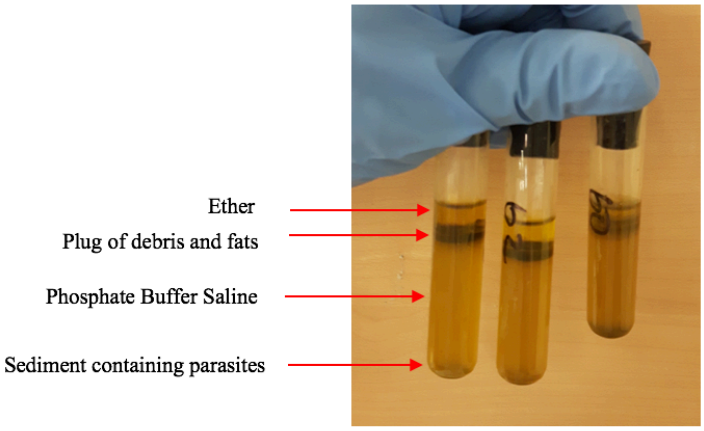

Figure 3.7.: PBS-ether sedimentation of cattle fecal specimen. Arrows pointing to four layers of separation post centrifugation.

First, 1-2 g of fecal specimen was transferred to a 10 ml test tube containing 5 ml of PBS. If the fecal specimen was liquid, approximately 1.5 ml was transferred to the test tube. To remove larger particles, the specimen was filtered through a sieve made out of medical gauze. The

resulting homogenate contained 7 ml of fecal material (30% [vol/vol] in PBS). Approximately, 2-3 ml of ether was added to the mixture. The mixture was centrifuged for 10 minutes at 500 x *g*. The resultant product was comprised of four layers, which included ether, a section of debris and fats, PBS and sediment containing potential parasites (Figure 3.7). A swab stick was carefully passed around the circumference of the tube to loosen the sheet of debris separating the two liquids. Then, the top three sections were discarded, permitting only the final sediment at the bottom. Last, 100 µl of nuclease-free water was added to the sediment and 5 µl of the suspension was transferred to a glass slide in preparation for MAF staining and microscopy. The remaining suspension of sediment was stored at -20 °C until DNA extraction.

3.3.3. MAF Staining and Microscopy

MAF staining and microscopy was performed to identify *Cryptosporidium* oocysts from cattle feces according to the CDC Modified Acid-Fast Staining Procedure (CDC, 2016b) with minor adjustments. The Ward's ® Chemistry Acid-Fast Stain Kit was used for the reagents, but staining followed guidelines provided in CDC's protocol (Table A.II., Appendix A).

First, the microscope slides containing 5 µl of sediment were air dried, then fixed with 99.8% methanol for 30 seconds. Fixation of the samples ensured that oocysts retained their morphological entity throughout the process (Ibrahim et al., 2016). Next, the slides were rinsed with distilled water and carbol fuchsin was added, then incubated at room temperature for 4 minutes. The slides were washed and decolorized with acid alcohol until they ran clear. Last, the slides were counterstained with methylene blue solution and incubated at room temperature for 2 minutes. The slides were then rinsed, dried and a coverslip was added.

The examination process started with 40× magnification objective to identify the presence of *Cryptosporidium* oocysts followed by confirmation using 100 × oil immersion objective. Scale bar was used to measure the diameter of oocysts. If a single oocyst appeared a pinkish-red colour with a diameter of approximately 4-8 µm, then the sample was considered positive. Two positive controls were used as reference to confirm detections: 1) positive control seeded into fecal specimen as described section 3.3.2.; and 2) *C. parvum* slide (VWR, Mississauga, Ontario).

3.4. ELISA Detection of *Cryptosporidium* Antigens from Cattle Feces and Microplate Reader Interpretation

Frozen cattle feces (*n*=119) were thawed in preparation for ELISA using the CRYPTOSPORDIUM TEST (Table A.IV., Appendix A). This kit was used in previous studies, which detected *Cryptosporidium* antigens from cattle from Belgium (Geurden, Berkvens, Geldhof, Vercruysse, & Claerebout, 2006a), India (Khan et al., 2010) and Zambia (Geurden et al., 2006b). The test uses monoclonal and rabbit polyclonal antibodies against *Cryptosporidium* oocysts, to form a complex with the parasites' antigens when present.

First, 400 µl of Diluent was transferred to a 2 ml microcentrifuge tube, then approximately 0.1 g (about the size of a small pea) of fecal specimen was added to the tube and thoroughly mixed by vortexing. If the fecal specimen was liquid, 100 ml was added. Second, 100 µL of Diluent was transferred to each test well of the Microassay Plate and a Pasteur pipette was used to transfer 1 drop of the fecal dilution to each well. The plate was sealed with a plastic adhesive sheet, which was incubated at room temperature for 1 hour. After incubation, the plate was inverted and knocked on paper towel to discard content from the wells. Each well was rinsed with 1X Wash

Solution five times, until the plate appeared cleaned. The plate was inverted and knocked on paper towel to remove the liquid. One drop of Conjugate was added to each well. The plastic adhesive sheet was seal over the plate and incubated at room temperature for 30 minutes. The same wash procedure was repeated five times. Two drops of Substrate were added to each well and incubated at room temperature for 10 minutes. Last, 1 drop of Stop Solution was added to each well, which changed the complexes to a yellow colour.

The intensity of yellow was an indicator of *Cryptosporidium* antigens. This was quantified using a Thermo Labsystems Opsys MR Microplate reader (Thermo Fisher, Walthan, MA). The absorbance was measured 450 nm and readings ≥0.150 were considered positive, according to the manufacturer's protocol.

3.5. Statistical Analysis

Standard deviation was calculated to indicate the extent of deviation of average biofilm thickness and roughness measurements, as described in section 3.1.4. Relative standard deviation (RSD) was calculated to determine whether standard deviation was small or large in comparison to the mean. This was applied to turbidity levels of each biofilm suspension, and subsequent *C. parvum* oocysts recovery efficiencies, as described in section 3.1.5.

%RSD is defined as follows:

$$\%RSD = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \quad (3)$$

In terms of *Cryptosporidium* detection from cattle feces, the proportions of positive detections between dairy and beef animals were evaluated. Differences in proportions of positive detections were assessed using the Chi Square Test, p-values. In addition, diagnostic agreement between MAF microscopy and ELISA antigen test was statistically evaluated. PCR was not included in this analysis due to low detection sensitivity in cattle feces, relative to MAF and ELISA techniques. Diagnostic (inter-rater statistical) agreement between the two techniques was estimated using Cohen's kappa (k) coefficient. Interpretation of the k coefficient determined whether agreement was due to chance or systematic agreement (i.e., consistent patterns).

k is defined as follows:

$$k = \frac{Po - Pe}{1 - Pe} \quad (4)$$

Where Po is the observed agreement amoung raters, and Pe is the expected agreement (i.e., hypothesized probability of agreement by chance). The k values were calculated by using the observed data to determine the probability of each observer randomly seeing each category. Interpretation of k values was performed using the Byrt (1996) classification scheme, summarized in Table 3.1.

Table 3.1.: Summary of Proposed Kappa Classification Scheme

Kappa Value	Degree of Agreement
0.00, or less	None
0.01-0.20	Poor
0.21-0.40	Slight
0.41-0.60	Fair
0.61-0.80	Good
0.81-0.92	Very Good
0.93-1.00	Excellent

Source: Byrt (1996)

3.6. PCR Detection of *Cryptosporidium*

3.6.1. 18S rRNA Conventional and Nested-PCR

PCR in conjunction with gel electrophoresis is the most widespread genotyping assay used for *Cryptosporidium* detection in research laboratories. Here, conventional PCR was performed for initial screening of *Cryptosporidium* from biofilms and cattle feces by amplifying fragments of the hypervariable region of the 18S rRNA gene. This was achieved by using CPB-DIAG primers, which targeted the 435 bp. Samples confirmed positive by conventional PCR were subjected to nested-PCR using primers specified in Table 3.2. Non-specific bands observed from conventional PCR products—within ±50 bp of the 435 bp target—were also subjected to nested-PCR, in order to ensure that they were true negatives.

In general, nested-PCR was performed to improve sensitivity and specificity of detection by

incorporating two sets of primers, which was suitable for detecting low number of oocysts typically found in environmental waters and fecal samples. Therefore, XIAO 1 and XIAO 2 nested primers were used to detect *Cryptosporidium* from cattle specimens, as described in Xiao et al. (1999). Whereas, KJL and CPB-DIAG nested primers were used to detect *Cryptosporidium* from biofilm suspensions, as described in Jellison et al. (2002).

Table 3.2.: Primers for 18S rRNA Conventional and Nested-PCR

Forward and Reverse Primers and Sequences	PCR	Target Gene	bp	References
CPB -DIAGF AAG CTC GTA GTT GGA TTT CTG **CPB -DIAGR** TAA GGT GCT GAA GGA GTA AGG	Conventional	18S rRNA	435	Johnson et al. (1995)
XIAO 1F TTC TAG AGC TAA TAC ATG CG **XIAO 1R** CCC ATT TCC TTC GAA ACA GGA	Nested (Outer)	18S rRNA	1325	Xiao et al. (1999)
XIAO 2F GGA AGG GTT GTA TTT ATT AGA TAA AG **XIAO 2R** AAG GAG TAA GGA ACA ACC TCC A	Nested (Inner)	18S rRNA	819-835	Xiao et al. (1999)
KLJ 1 CCACATCTAAGGAAGGCAGC **KLJ 2** ATGGATGCATCAGTGTAGCG	Nested (Outer)	18S rRNA	1056	Jellison et al. (2002)
CPB -DIAGF AAG CTC GTA GTT GGA TTT CTG **CPB -DIAGR** TAA GGT GCT GAA GGA GTA AGG	Nested (Inner)	18S rRNA	435	Johnson et al. (1995)

The 18S rRNA was the most appropriate gene target selected for conventional and nested-PCR because *Cryptosporidium spp.* were detected from a diverse pool of environmental samples. Moreover, the 18S rRNA gene has the richest sequencing database available for *Cryptosporidium* compared to any other gene targets including: *Cryptosporidium* oocyst wall protein (COWP); Thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1); and Heat shock protein 70 (hsp70) etc. (Xiao & Ryan, 2008). Therefore, the high degree of sequence homology presented in the 18S rRNA enabled the use of primers that could amplify a wide range of *Cryptosporidium spp.* isolated from environment sources (Kimbell, Miller, Chavez, & Altman, 1999).

3.6.2. Gp60 Nested-PCR

Positive *Cryptosporidium* samples were further characterized using a nested-PCR assay, which amplified the variable region of the gp60 gene using primers specified in Table 3.3. Different combinations of these primers have been incorporated in previous studies to identify subtypes of *C parvum* and *C. hominis* from cattle feces (Alves et al., 2003; Hatalová, Valenčáková, & Kalinová, 2017) and wastewater sources (Feng, Li, Duan, & Xiao, 2009; Glaberman et al. 2002). Overall, gp60 characterization of *Cryptosporidium* was an important component, as it revealed the epidemiological relevance of positive detections.

Table 3.3.: Primers for gp60 Nested-PCR

Forward and Reverse Primers and Sequences	PCR	Targeted Gene	bp	References
Gp60F1 ATG AGA TTG TCG CTC ATT ATC G **Gp60R1** TTA CAA CAC GAA TAA GGC TGC	Nested (Outer)	gp60	980-1000	Strong, Gut, and Nelson (2000)
AL3532 TCC GCT GTA TTC TCA GCC **AL3535** GGA AGG AAC GAT GTA TCT	Nested (Inner)	gp60	800-850	Peng et al. (2001) and Alves et al. (2003)

3.6.3. Oocysts Rupture and DNA Extraction

Oocyst rupture and DNA extraction was performed on the cattle feces and biofilm samples using the QIAamp® Fast DNA Stool Mini Kit and reagents listed in Table A.V. in Appendix A. Two positive controls were assessed alongside the samples. First, to rupture oocysts from the biofilm suspensions, 200 µl of each packed pellet, previously isolated via IMS was transferred to a 1.5 ml microcentrifuge tube. Then, 50 µl of 20% (w/v) suspension of Chelex®100 Resin was added to the mixture to reduce potential degradation of DNA. The samples were then subjected to seven cycles of freezing and thawing at −70°C for 15 minutes and 70°C for 15 minutes. The same procedure was repeated for the cattle specimens, which were previously concentrated via PBS-ether sedimentation. The remaining extraction process was performed using the QIAamp® Fast DNA Stool Mini Kit as per the manufacturer's protocol with minor adjustments.

Briefly, the sample pellets were suspended in 1 ml InhibitEX Buffer and incubated at 70°C for 5 minutes. The samples were centrifuged at 20,000 x g to pellet the particles. The supernatant was transferred to a new 1.5 ml centrifuge tube containing 200 µl Buffer AL, and 15 µl of proteinase

K was added to the lysate. Next, the lysate was incubated at 70°C for 10 minutes and 200 µl of 100% ethanol was added. The entire lysate was transferred to the QIAamp spin column and centrifuged at 20,000 x g for 1 minute. This process was repeated 2-3 times until the full lysate was filtered through the QIAamp spin column. Next, 200 µl of Buffer AW1 was added to the QIAamp spin column, which was centrifuged at 20,000 x g for 1 minute. Subsequently, 200 µl of Buffer AW2 was added to the lysate and centrifuged at 20,000 x g for 3 minutes. Last, 50 µl of Buffer ATE was added to the QIAamp spin column, then centrifuged for 1 minute to elute the DNA. Notably, the final three Buffers added to the lysate was reduced from the original protocol to concentrate the *Cryptosporidium* DNA, as it was anticipated to be very low from environmental sources (Jiang et al., 2005). The extracted DNA samples were stored in a −20°C freezer until PCR.

3.6.4. Spectrophotometric Analysis

Spectrophotometric analysis was performed to determine optimal primer dilution prior to genotyping assays. It was also used to evaluate the purity and concentration of DNA from purified PCR products. The NanoDrop ND1000 (Thermo Fisher, Walthan, MA) measured the solutions' absorbance spectrum between 220 and 330 nm to quantify concentration (ng/µl). Also, two absorbance ratios, A_{260}/A_{280} and A_{260}/A_{230} were recorded to evaluate purification.

First, dry primers were diluted in molecular grade water. NanoDrop ND1000 was erased using a blank cuvette. The solutions were inserted in the machine, and absorbance spectrum, ratios and concentration were recorded. Approximately, 40 ng/µl was considered to be optimal concentration of 5 µM (working dilution) primers (Xiao & Ryan, 2008). The primers were further diluted if they did not fall within this range.

Second, the purity and concentration of DNA from PCR products were evaluated in the same manner as the primers. According to Themo Scientific (2009) A_{260}/A_{280} and A_{260}/A_{230} ratios indicate the presence of protein, phenol and/or other contaminants that absorb strongly at or near 280 and 230 nm, respectively. Ratios between 1.8 and 2.0 for A_{260}/A_{280} were considered pure. Similarly, ratios between 1.8 and 2.2 for A_{260}/A_{230} were considered pure (Sambrook& Russell, 2006; Thermo Scientific, 2009). Last, DNA of samples <20 ng/µl were considered very low (Genomics Core Facility, n.d.), thus thermocycler conditions were adjusted for processing cattle DNA by increasing the number of cycles to 50 (refer to section 3.6.6). It is important to acknowledge that increasing the number of PCR cycles, may have generated more undesired mutations, resulting in primer dimers and a greater chance of false positives. However, this modification was necessary after multiple attempts to increase the concentration of extracted DNA. This included: modifying the DNA extraction procedure by decreasing the Buffer ATE volume for the final suspension step (section *3.6.3.*); lowering the annealing temperature after running a temperature gradient test (section *3.6.6.*); and testing alternative master mixes to determine most effective.

3.6.5. Preparation of Master Mixes

FastStart™ Taq DNA Polymerase master mix, dNTPack (Table A.VI, Appendix A) was used to verify replicates of *Cryptosporidium* positive controls (*n*=3), which were thereafter used in subsequent genotyping assays (refer to Figure D.III, Appendix D. for electrophoresis images). Positive controls derived from two sources: 1) human stool sample—clinically diagnosed positive for *C. meleagridis* in 2012. The use of human stool was approved by the UHWI/UWI/Faculty of Medical Sciences (FMS) Ethics Committee. 2) cattle fecal sample

C8Cw, which was confirmed positive via MAF staining and ELISA as described in sections 3.3. and 3.4. Also, a negative control was included with every reaction.

Conventional and nested-PCRs, targeting the 18S rRNA, were performed with FastStart™ master mix in the Department of Microbiology laboratory at UWI. All reagents used to create the master mix were prepared under a biosafety cabinet, which was cleaned immediately before usage to avoid contamination. A final reaction volume of 50 µl was prepared for each sample. The concentration and volume per reaction tube are provided in Table 3.4.

Table 3.4.: Preparation of Master Mix with FastStart™ Taq DNA Polymerase, dNTPack for 18S rRNA Conventional and Nested-PCR

Reagents	Concentration	Volume per reaction tube (µl)
PCR Buffer	10X	5
Nuclease-free water	-	33
$MgCl_2$	25 nM	3
dNTPs	20 mM	1
Forward Primers	10 µM	1
Reverse Primers	10 µM	1
Taq Polymerase	5 U/µl	1
Positive Control DNA/ Primary PCR Product	-	5
Final Volume		50

Note: the master mixes prepared for conventional and nested-PCR were identical

AccuStart™ II PCR ToughMix master mix (Table A.VI, Appendix A) was used for 18S rRNA conventional and nested-PCR detection of *Cryptosporidium* from biofilm and cattle fecal samples. The PCR assays were performed by The Hospital for Sick Children, The Centre for Applied Genomics (TCAG), Genetic Analysis Facility, Toronto, ON. A final reaction volume of 10 µl was prepared for each sample. The concentration and volume per reaction tube are provided in Table 3.5.

Table 3.5.: Preparation of Master Mix with AccuStart™ II PCR ToughMix for 18S rRNA Conventional and Nested-PCR

Reagents	Concentration	Volume per reaction tube (µl)
AccuStart™ II PCR ToughMix	2X	5
Nuclease-free water	-	2
Forward Primers	5 µM	1
Reverse Primers	5 µM	1
Extracted DNA/ Primary PCR Product	-	1
Final Volume		10

Note: the master mixes prepared for conventional and nested-PCR were identical

GoTaq® DNA polymerase (Table A.VI, Appendix A) was used for gp60 nested-PCR detection of *Cryptosporidium* from positive biofilm and cattle fecal samples. This was performed prior to sequencing by the CDC, Division of Foodborne, Waterborne and Environmental Diseases,

Atlanta, Georgia. A final reaction volume of 50 µl was prepared for each sample. The concentration and volume per reaction tube are provided in Table 3.6.

Table 3.6.: Preparation of Master Mix with GoTaq® DNA polymerase for gp60 Nested-PCR

Reagents	Concentration	Volume per reaction tube (µl)	
		Primary Master Mix	Secondary Master Mix
PCR Buffer 3 (contain $MgCl_2$)	1X	5	5
Nuclease-free water	-	35.35	33.85
dNTPs	1.25 mM	4	4
Forward Primers	10 µM	1.25	2.5
Reverse Primers	10 µM	1.25	2.5
BSA	10 mg/ml	2	0
Taq Polymerase 2	5 U/µl	0.15	0.15
Extracted DNA/Primary PCR Product	-	1	2
Final Volume		50	50

3.6.6. Temperature Gradient and Thermocycler Conditions

The PCR assays described above have widespread applications for *Cryptosporidium* detection; however, there are reported drawbacks including reduced sensitivity and specificity from environmental samples, as mentioned in section 1.6.1. Given these limitations, optimization of

18s rRNA primers was critical to perform prior to PCR. Thus, a temperature gradient was conducted, which determine band intensity at various annealing temperatures. These results helped to inform thermocycler conditions. For CPB-DIAG primers, the annealing temperatures ranged from 52.5 to 65°C and differed by ±2.5°C increments. Similarly, XIAO nested primers ranged from 42.5 to 55°C and differed by ±2.5°C increments.

PCR amplifications were performed with either the SimpliAmp™ Thermal Cycler or GeneAmp® PCR System 9700. For conventional PCR, the thermocycler conditions were as follows: an initial denaturation step at 95°C for 1 minute, then 35 cycles at 95°C for 15 seconds, 52.5°C for 15 seconds, and 72°C for 30 seconds with a final extension of 72°C for 7 minutes. For nested-PCR, the first round amplified the external PCR-products, which were used as templates for the nested reaction. The thermocycler conditions were relatively the same. The only difference was that the annealing temperature was increased to 55°C, as described in Xiao et al. (2001). For all cattle samples, the number of PCR cycles were increased to 50 given the presence of low DNA. Finally, the thermocycler conditions for gp60 nested-PCR were as follows: an initial denaturation step at 94°C for 45 seconds, then 35 cycles at 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 60 seconds with a final extension of 72°C for 7 minutes. The primary and secondary rounds of PCR were identical.

3.6.7. Gel Electrophoresis and Visualization

PCR products were visualized by gel electrophoresis using reagents specified in Table A.VI., Appendix A. The positive and negative control PCR products were ran on 1.5% agarose gel in TAE buffer. The gel was prepared by dissolving 1.2 g of Ultra-Pure™ agarose in 100 ml of 0.5X TAE buffer in an Erlenmeyer flask. The solution was heated in a microwave to a full boiling

point. Once cooled, 0.5 ml of working stock Ultra-Pure™ ethidium bromide was added. The solution was thoroughly mixed and poured into a cast containing a comb row, which created the wells. The gel was allowed to harden for approximately 1 hour, then submerged into an electrophoresis tank containing 0.5X TAE buffer. Next, 3 µl of DNA gel loading dye (6X) for each PCR reaction was spotted on parafilm and 20 µl of the PCR products were added to the loading dye. The mixture was transferred to the wells and 10 µl of PCR 50 - 2000 bp marker was added to the first well. The electrophoresis was carried out at 100 V for 1.5 hours. Bands of predicted size were visualized under UV light using a transmitter box. Gp60 PCR products were also ran on agarose gels in TAE buffer.

Biofilm and cattle PCR products were subjected to capillary electrophoresis using the QIAxcel DNA Fast Analysis Kit (Table A. IV, Appendix A). Ready-to-use gel cartridges, buffers, and a 50 bp alignment maker was loaded into the instrument, along with microcentrifuge tubes containing PCR products. The process profile was selected, and the system was ran for 1.5 hours. Bands of the predicted size were visualized with the QIAxcel ScreenGel Software (Qiagen, Hilden, Germany). The DNA band intensities of all positive detections were ranked according to the following scheme: ++++ = very strong, +++ = strong, ++ = moderate, + = weak intensity, and – = no visualization (Adamska et al., 2012). This intensity scheme was referenced with positive control, C8Cw shown in Figure 4.5c.

3.6.8. Purification of Positive PCR products

Purification of PCR products was performed partially at Ryerson University, and by The Hospital for Sick Children, genomic services. In both cases, the QIAquick® PCR Purification Kit (Table A.VII., Appendix A) was used as per the manufacturers protocol with minor

adjustments. A 5:1 volume of Buffer PB was added to each PCR product and mixed. The product mixture was added to a Qiaquick spin column and centrifuged for 1 minute. Then, 1 ml of Buffer PE was added to the spin column and centrifuged twice for 1 minute. Between each centrifugation, the flow-through solution was discarded. The Qiaquick spin column was placed in a new 1.5 ml centrifuge tube, and 30 µl of Buffer EB was added, followed by incubation for 1 minute and centrifugation for 1 minute. The DNA collected in the microcentrifuge tube was stored at −20 °C before submitting for sequencing.

3.7. Gene Sequencing and Phylogenetic Analysis of *Cryptosporidium spp.* and Subtypes

3.7.1. Sequencing 18S rRNA and gp60 PCR Products

Sequencing of 18S rRNA PCR products was carried out by The Hospital for Sick Children, genomic services with primers previously described in Table 3.2. Likewise, sequencing of gp60 PCR products was performed by the CDC with nested primers, AL3532/35 and gp60 described in Table 3.3. In both labs, the BigDye® Terminator v3.1 Cycle Sequencing Kit was used for the procedure (Table A.VIII., Appendix A). Purified amplicons were directly sequenced in the forward and reverse directions with either the Applied Biosystems 3730XL DNA Analyzer or Applied Biosystems 3130XL Genetic Analyzer.

3.7.2. NCBI Basic Local Alignment Search Tool (BLAST) and Development of Phylogenetic Tree

NCBI basic local alignment search tool (BLAST) was used to compare readable 18S rRNA sequences to the GenBank database. The online archive consisted of previously submitted

Cryptosporidium sequences, most of which were from the same locus. Sequences were downloaded into ClustalX in FASTA format and aligned in "Multiple Alignment Mode", then manually trimmed. The output of aligned sequences produced gaps, which were included in the phylogenetic analysis, as it provides informative data about evolutionary paths/relationships (Evans, & Warnow, n.d.).

The aligned sequences were downloaded into MEGA7 tree-building software and compared to previous submissions using the BLAST algorithm. If multiple clones from a single sample were sequenced with 95 to 100% identity, then the most homologous match was brought into MEGA7. Finally, the phylogenetic tree was constructing using the clustering algorithm, Neighbour-Joining, which evaluated the dissimilarity between aligned sequences (Moura, 2010). Various *Cryptosporidium spp.* were added to the tree, along with an outgroup organism, Eimeria tenella (accession number AF026388) that served as reference for determining evolutionary relationships within the ingroup. Last, a bootstrapping maximum likelihood method with 1000 replicates of original consensus alignments was used to access the accuracy of clustering results. Bootstrap values 50% or higher were included in the tree; however, the topology of the branch was considered "correct" only if the value of an interior branch was greater than 95% (MEGA Software, 2011).

3.8. ARC GIS Mapping

ARC GIS mapping was conveyed to provide a visual representation of sampling allocations. Two-dimensional maps were created using ARCMap10 (ESRI Canada Limited). The basemap of Jamaica pre-existed in the software and was used to allocate sampling sites displayed in Figures 3.3. and 3.6. To outline terrestrial boundary, an image of the Black River watershed (Figure 3.8.)

was downloaded from Jamaica's National Environment and Planning Agency [NEPA] (2003).

The on-screen digitizing tool was used to convert the image into vector data by tracing its features onto the basemap. This tool was also used to convert the river network to a bright blue colour. A sampling location layer was added to the basemap by creating an input table in Microsoft Excel, which specified biofilm and cattle farm locations along with corresponding coordinates. The coordinates, in decimal degree latitude and longitude format, were retrieved at the same time of sample collection using the Maps Coordinate App. The table was exported as a .dbf file and the points were geocoded onto the basemap. A topography layer shown in Figure B.I in Appendix B was created using 20 m contour data, which was downloaded from DIVA-GIS in the form of a Digital Elevation Model (DEM). Scale bar, compass and legend were added to the maps.

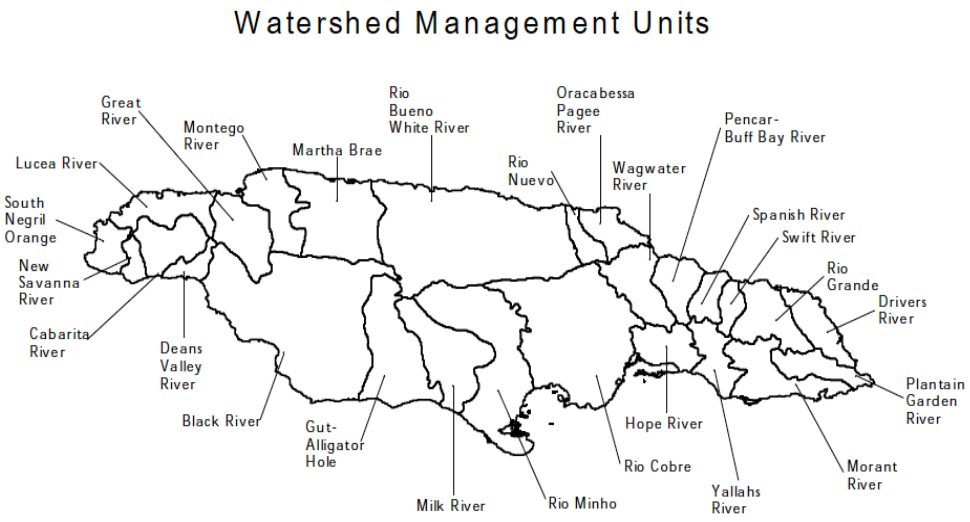

Figure 3.8.: Map used for outlining terrestrial boundary of the Black River watershed in ARCScene10 and ARCMap10. Source: NEPA (2003).

Three-dimensional contour maps shown in Figure B.II. in Appendix B. was generated using ARCScene10 (ESRI Canada Limited). A raster image of elevation values was added to the scene layer. The same 20 m contour DEM used in ARCMap10 (ESRI Canada Limited) was brought into the software, and the base height was adjusted to reflect contour value. The sampling location layer was geocoded onto the scene layer. An image of the Black River network shown in Figure 2.3. was downloaded from Björk (2013). The on-screen digitizing tool was used to trace the river network onto the scene layer. Compass was added to the maps.

Chapter 4: Results

4.1. Method Validation and Performance of the *Biofilm Sampler* Technique

4.1.1. Biofilm Thickness and Roughness

During the spring of 2016, a pilot study was performed in which biofilms were developed on 4 polycarbonate-slides and 1 glass reference-slide using a customized *biofilm sampler*. The purpose was to determine whether biofilms could reach an optimal level of maturation that could entrap and retain *Cryptosporidium* oocysts. After 3-weeks, a thin strip of biofilm formation was observed on all slides (Figure 4.1.). Three-dimensional, confocal images of biofilm segments were captured (n=32) from half of each polycarbonate-slide and the entire glass reference-slide (approximate area of 19.5 cm^2 from both substrate). The images were subsequently analyzed via COMSTAT2 to determine biofilm thickness and roughness. Findings are provided in Table 4.1., with complete data available in Table C.V., Appendix C.

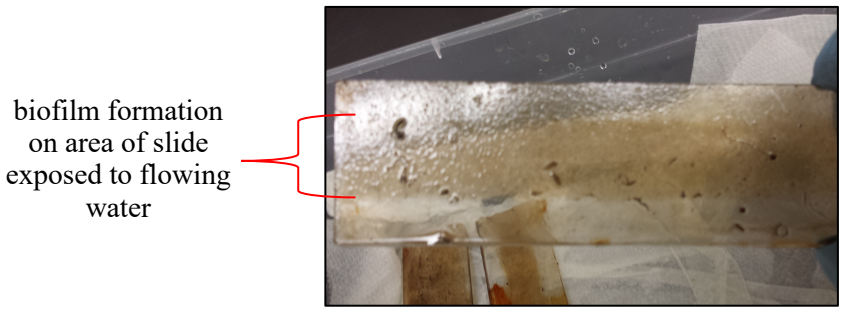

Figure 4.1.: Thin layer of biofilm formation on polycarbonate slides after 3-weeks submerged under water in *biofilm sampler*.

Average biofilm thickness measured from the polycarbonate-slides ranged from 48.33 to 82.53 µm. While maximum average thickness was 95.63 to 115.13 µm. In contrast, biofilms developed on the glass substrate had an average thickness of 22.85 µm and a maximum average thickness

of 29.21 µm. Furthermore, microbial colonization was visibly thicker on the polycarbonate substrate compared to the glass reference-slide, as shown in Figure 4.3c. and 4.3d.

With regards to biofilm roughness, mean R_a* of the polycarbonate-slides were between 1.63 and 1.78, indicating that biofilms were variable in terms of thicknesses. These observations are reflective of biofilm patchiness or heterogenous formation (Figure H, Appendix H). In contrast, mean R_a* measured from the glass reference-slide was 0.49, which indicates that biofilm formation was evenly distributed.

Table 4.1.: COMSTAT2 Analyses of Biofilm Thickness and Roughness Measured from Polycarbonate-Slides and a Glass Reference-Slide

Biofilm ID	Average Thickness (µm)[a]	Maximum Thickness (µm)[a]	Roughness Coefficient[a]
Bply1[b]	48.33 ± 29.41	96.88 ± 27.08	1.76 ± 0.21
Bplyt2[b]	52.78 ± 27.47	95.63 ± 24.41	1.73 ± 0.26
Bply3[b]	82.53 ± 17.83	115.13 ± 14.89	1.78 ± 0.25
Bply4[b]	60.47 ± 32.55	100.13 ± 17.88	1.63 ± 0.20
BglsREF[c]	22.85± 4.54	29.21 ± 6.81	0.49 ± 0.27

[a] Mean ± S.D. (*n*=8)
[b] polycarbonate substrate
[c] glass substrate

4.1.2. Recovery Efficiency of C. parvum Oocysts Seeded into Biofilms

The remaining biofilm-halves (*n*=4) were suspended in water, then seeded with *C. parvum*

oocysts. Recovery efficiency was then determined by IMS-IFA microscopy, using REF coverslips to facilitate quantification (Figure 4.2.). As shown in Table 4.2., mean recovery ranged between 23 and 68%, with a seed dose of 2422 ± 138 oocysts and a mean turbidity of 548.1 ± 25.95 NTU. Complete data available in Table C.VI. of Appendix C. Therefore, oocysts recoveries were variable even though the seed dose and turbidity levels were fairly consistent, and the biofilms were developed simultaneously in a single *biofilm sampler*.

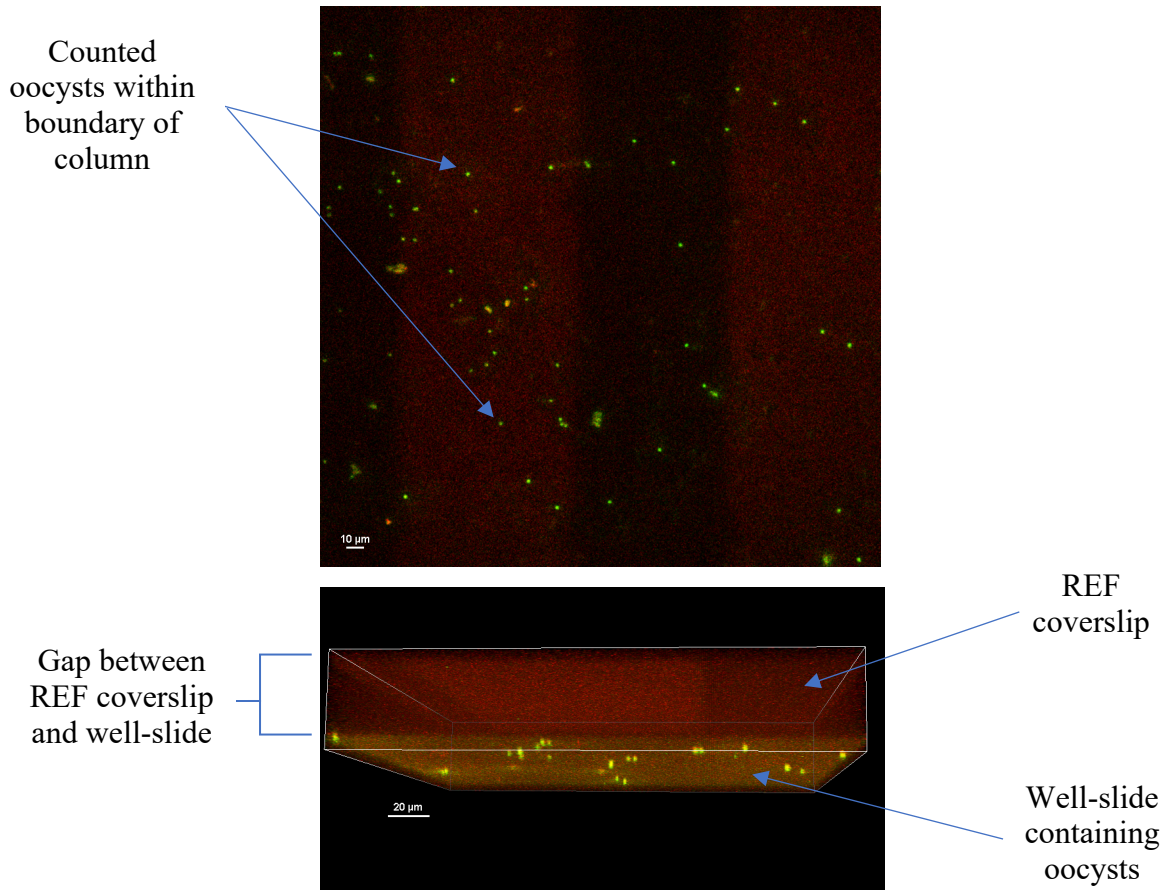

Figure 4.2.: Quantification of *C. parvum* oocysts from biofilm using IMS-IFA and REF coverslips displayed in 2D and 3D. Technique determined recovery efficiency of oocysts from biofilm suspensions. Number of oocysts (green) were counted by scanning up and down individual columns (red) within boundary of the well-slide. Magnification 40X.

Table 4.2.: IMS Recovery of *C. parvum* Oocysts from Biofilm Suspensions

Biofilm ID	Turbidity of 4-ml Concentrate (NTU)[b]	Recovery (%)[a]
Bply1	544.1 ± 4.54	68.7 ± 5.1
Bply2	554.1 ± 4.84	35.1 ± 2.7
Bply3	549.6 ± 4.30	43.9 ± 14.4
Bply4	544.3 ± 4.99	23.9 ± 5.6
Mean ± RSD	**548.1 ± 4.73**	**42.9 ± 18.45**

[a] Mean ± RSD (*n*=4)
[b] Mean ± RSD (*n*=10)
Seed dose of 2422±138 oocysts/50 µl

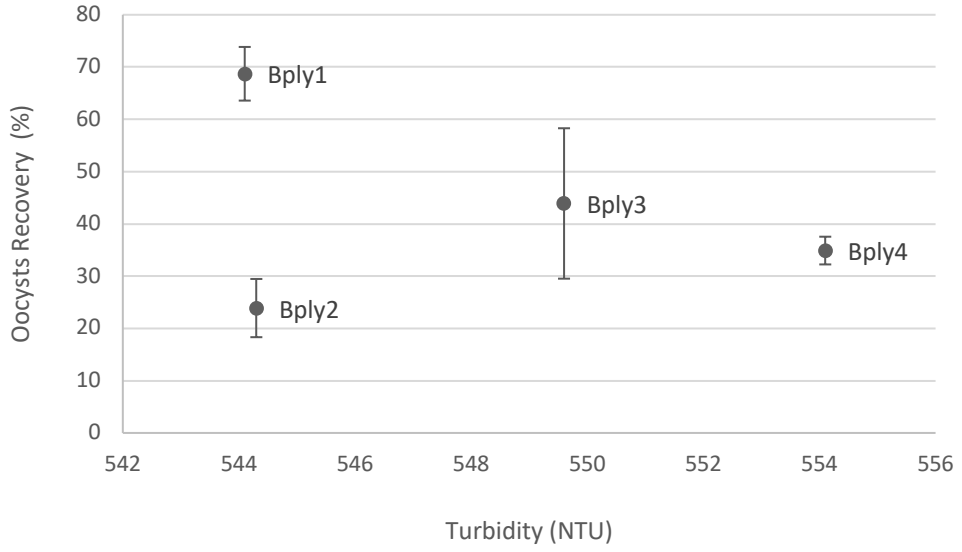

Figure 4.3.: Relationship between turbidity and recovery efficiency of *Cryptosporidium* oocysts seeded into environmental biofilms. Error bars indicate the S.D. between microscope-slide replicates (*n*=4) for each biofilm suspension.

Furthermore, the S.Ds. of oocysts recovery from biofilm suspensions, Bply1, Bply2, Bply3 and Bply4 were 5.1, 2.7, 14.4 and 5.6%, respectively. Therefore, with the exception of Bply3, there were no significant differences between replicated microscope slides that were derived from a single biofilm (Figure 4.3.). Variability of oocyst recovery was dependent on the uniqueness of biofilm composition, rather than inconsistencies between slide replicates.

4.2. Optimization of PCR Assays

4.2.1. Spectrophotometric Analysis of Primers and Purified DNA

Spectrophotometry was performed to evaluate the concentration and purity of primers and purified DNA from selected biofilm and cattle samples. Original NanoDrop readings are available in Table C.VII., Appendix C.

Primer concentration and absorbance ratios were determined after appropriate dilutions were made from 100 µM stock primers. As shown in Table 4.3., concentration of the final working dilutions ranged between 28 and 42 ng/µl, which is consistent with ~40 ng/µl, as recommended in Fayer and Xiao (2008).

DNA concentration was determined from selected biofilm and cattle samples (Table 4.4.). Surprisingly, biofilm samples C2Bio and D3Bio had a DNA concentration of 319.11 and 267.13 ng/µl, respectively. This is significantly higher than cattle sample C8Cw, which had a concentration of 10.57 ng/µl. Figure 4.4. shows the absorbance spectrum, illustrating the vast difference between *Cryptosporidium* DNA extracted from the two sample types.

Table 4.3.: Concentration and Purity of Primers

Primer	100 µM Stock Primers			5 µM Working Dilution Primers		
	Conc. (ng/µl)	**A_{260}/A_{280} ratio**	**A_{260}/A_{230} ratio**	**Conc. (ng/µl)**	**A_{260}/A_{280} ratio**	**A_{260}/A_{230} ratio**
CPB-DIAG Forward	642.31	1.53	1.41	35.67	1.52	2.63
CPB-DIAG Reverse	1125.65	2.06	2.71	42.12	1.54	2.14
KLJ Forward	718.15	1.53	2.31	37.89	1.51	2.19
KLJ Reverse	639.58	1.53	2.22	36.54	1.19	2.17
XIAO 1 Forward	552.59	1.84	2.39	28.74	1.62	2.50
XIAO 1 Reverse	596.40	1.70	2.10	30.05	1.49	2.22
XIAO 2 Forward	736.67	2.06	2.93	40.76	1.94	2.88
XIAO 2 Reverse	630.25	2.18	2.30	34.18	1.91	2.32

Table 4.4.: Concentration and Purity of Selected DNA

Sample ID	Concentration (ng/µl)	260/280 ratio	260/230 ratio
C8Cw[a]	10.57	2.65	0.07
C17Cw[b]	0.63	0.63	0.69
D3Bio[c]	267.13	1.90	2.24
C2Bio[c]	319.11	1.89	2.27
DNA ctrl[d]	90.32	1.92	1.77
NFW[e]	0.031	-1.67	2.67

[a] DNA extract from *Cryptosporidium*-positive cattle feces
[b] DNA extract from *Cryptosporidium*-negative cattle feces
[c] DNA extract from *Cryptosporidium*-positive biofilms
[d] Positive Control DNA
[e] Negative Control DN

With regards to purity, the A_{260}/A_{280} and A_{260}/A_{230} ratios of sample C8Cw were 2.65 and 0.07, respectively. These values do not fall within the acceptable "pure" range (between 1.8 and 2.2), as previously described in section 3.5.4. Therefore, excessive contaminants were present in sample C8Cw, which were anticipated to be present in the remaining cattle samples. In contrast, the A_{260}/A_{280} and A_{260}/A_{230} ratios of samples C2Bio and D2Bio were 2.27 and 2.24, respectively; thus, DNA from biofilms were very pure. Overall, these results suggest that *Cryptosporidium* DNA purified from the biofilm samples were significantly more concentrated and contained less PCR inhibitors compared to the cattle feces.

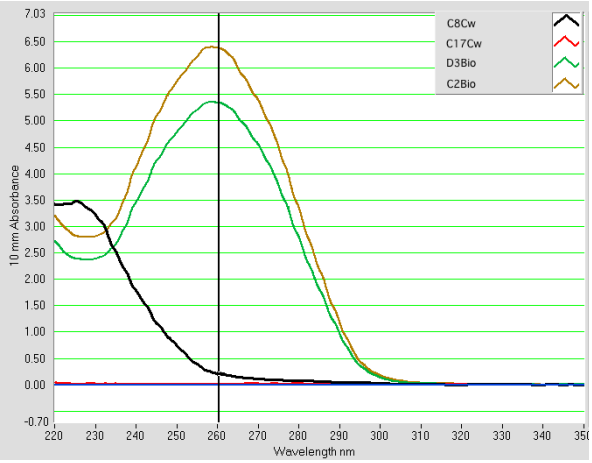

Figure 4.4.: NanoDrop Data Viewer- absorbance spectrum of *Cryptosporidium* DNA from cattle and biofilm samples. Absorbance at 260 nm is significantly lower for *Cryptosporidium*-positive cattle sample (C8Cw) compared to biofilm samples (D3Bio and C2Bio). *Cryptosporidium*-negative sample (C17Cw) shows no absorbance.

4.2.2. Temperature Gradients and Electrophoresis

Temperature gradients utilizing DNA from cattle sample C8Cw were performed with the AccuStart™ II PCR ToughMix and four different primer sets. Electrophoresis images are displayed in Figure 4.5.

It was determined that the optimal annealing temperature for CPB-DIAG primers was 52.5°C, which is slightly lower than previously described (55°C) in Johnson et al. (1995). At 52.5°C, CPB-DIAG primers showed a weak DNA band at the ~435 bp with non-specific binding present at <100 bp (Figure 4.5a.). Similarly, the optimal annealing temperature for KLJ primers, was 52.5°C, which is approximately the same as previously described (53°C) in Jellison et al. (2002). At 52.5°C, KLJ primers showed a very strong DNA band at the ~1056 bp, with no non-specific binding at <100 bp (Figure 4.5b.). With regards to XIAO1 and XIAO2 primers, targeting the 1325 bp and 835 bp, the optimal annealing temperature was 55°C, as recommended by Xiao et al. (1999). At 55°C, the nested primers showed very strong DNA bands with minimal to no non-specific binding at <100 bp (Figure 4.5c.).

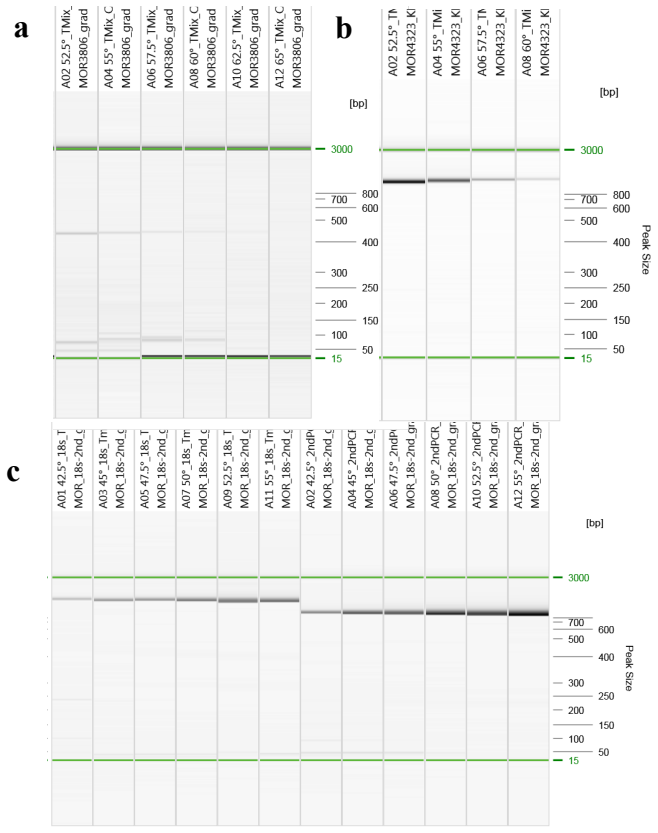

Figure 4.5.: Electrophoresis images from temperature gradients using selected CPB-DIAG (a), KLJ (b), and XIAO1 and XIAO2 (c) primers targeting the 18S rRNA. Temperature gradients were performed on extracted DNA from cattle sample C8Cw using AccuStart™ II PCR ToughMix.

4.3. Detection of *Cryptosporidium* from Biofilms

4.3.1. IFA Detection

IFA microscopy was performed prior to PCR, to detect *Cryptosporidium* oocysts from biofilm samples. This is the first available research to investigate the occurrence of *Cryptosporidium* in surface water in Jamaica. Thus, it was important to perform initial screening to determine the pathogen's presence within the geographic location. A total of 24 biofilm-slides were collected from the river network in July 2016 and February 2017. Bright-field IFA microscopy with oil immersion and DAPI was used to analyze the biofilm suspensions for *Cryptosporidium* oocysts, according to EPA Method 1623. Seventy-eight replicated microscope slides were examined from Sites A, B, C, D, and E.

Morphological characteristics of oocysts including size, shape, outer-wall distinctiveness, and apple-green fluorescence was found to be consistent with those of *Cryptosporidium spp.* Oocysts size was predominantly 4-6 µm in diameter (Figure 4.6.), although some appeared as large as 7 µm.

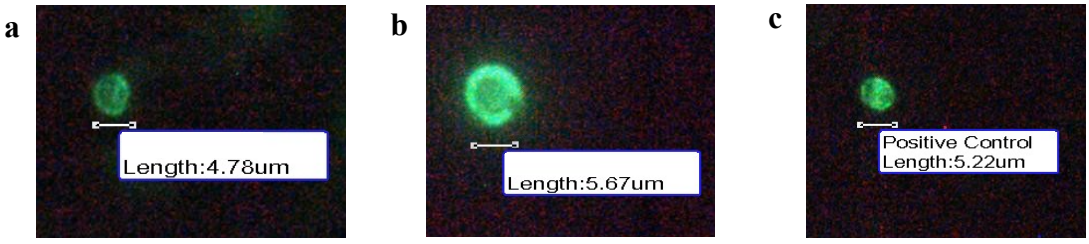

Figure 4.6.: *Cryptosporidium* oocysts detection from biofilms (a, b) and positive control (c) by IMS-IFA. The Crypt-a-Glo™ Comprehensive Kit was used for immunofluorescence staining of oocysts, as recommended by EPA Method 1623. Magnification, 100X with immersion oil.

As shown in Table 4.5., every sampling site was positive for *Cryptosporidium* oocysts in at least one microscope-slide replicate. Nine (11.5%) were positive by IFA with DAPI-visible nuclei,

whereas 14 (17.9%) were positive by IFA only. The frequency of oocysts detection was lowest at Site E, with 1 of 16 (6%) samples positive for *Cryptosporidium* by IFA only. Site C had the highest frequency of detection, with 6 of 15 (40%) samples positive for *Cryptosporidium* by IFA, of which 4 (27%) were positive with DAPI-visible nuclei.

Table 4.5.: IFA Detection of *Cryptosporidium* Oocysts from Biofilms (Sites A-E)

	No. of IFA positive detections (No. of slide replicates)	
Site Ref. (Region)	**Outer Shell**	**DAPI-Visible Nuclei**
Site A (Middle Quarters)	5 (16)	3 (16)
Site B (Cataboo)	1 (15)	1 (15)
Site C (Black River Capital)	6 (15)	4 (15)
Site D (Lacovia)	1 (16)	1 (16)
Site E (Bartons)	1 (16)	0 (16)
Total	**14 (78)**	**9 (78)**

4.3.2. Nested-PCR Detection and Correspondence with IFA

Following IFA, biofilms were analyzed for *Cryptosporidium* in replicates of 4 by nested-PCR. Initial samples collected in July 2016 from Sites A and B were not processed due to miscommunication of long-term storage at UWI, in which the samples were discarded. Figure 4.7. shows the electrophoresis images of the primary and secondary PCR products from Sites C, D and E. In the primary round, there were no positive detections, as revealed by the absence of amplification at the 1056 bp. When the primary PCR products were employed as templates in the secondary round, visible bands appeared at the ~435 bp. Second-round amplification was also observed in the positive control, while no amplification was observed in the negative control.

It was determined that 4 of 12 biofilm samples were positive for *Cryptosporidium*; one sample was from Site C, and 3 samples were from Site D. There were no positive detections from Site E, located in the upper reaches of the river. Positive detections were only derived from the intersecting region of the Upper and Lower Morass (Site C), as previously shown in Figure 3.3., as well as near to the river outlet (Site D).

The correspondence between *Cryptosporidium* detection by means of IFA and nested-PCR was evaluated. Findings are provided in Table 4.6. Positive PCR products were obtained from 2 biofilm samples (C2Bio and D4Bio) that were also confirmed positive by IFA. Likewise, PCR detection was obtained from 2 IFA-negative samples (D1Bio and D3Bio). Results show inconsistencies between some IFA and PCR detections. In total, 6 of 12 samples were confirmed positive by either method.

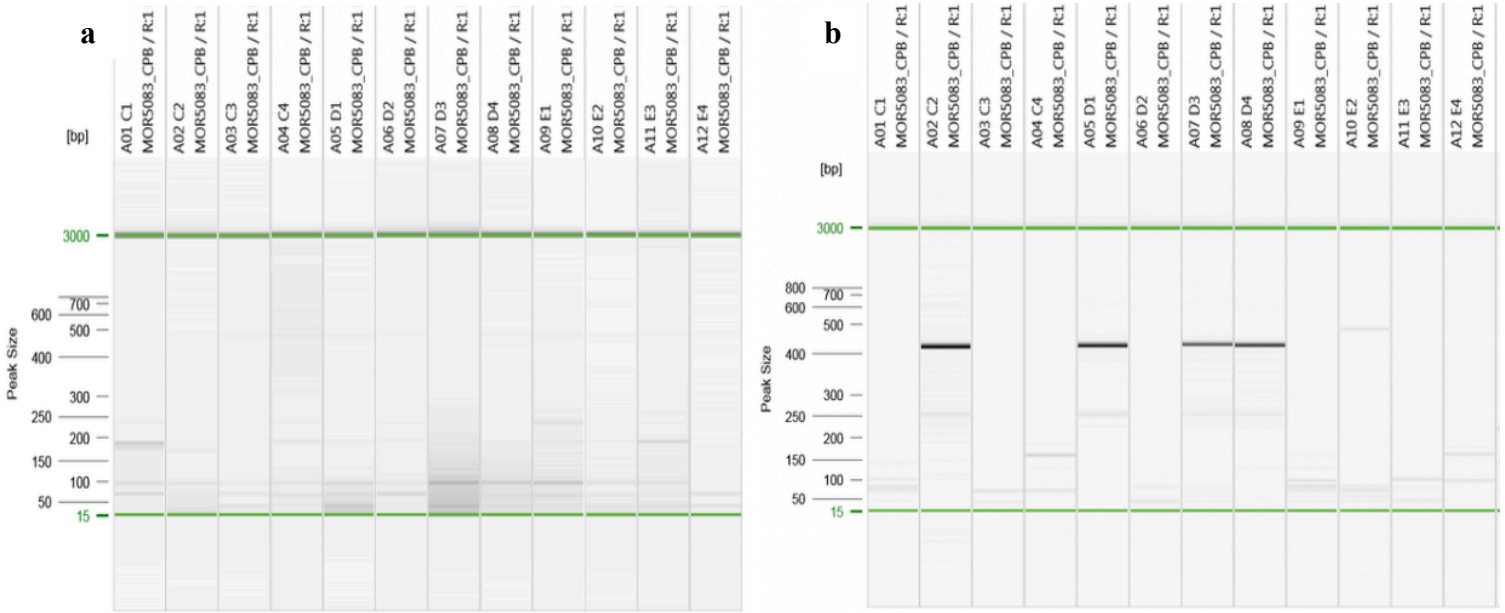

Figure 4.7. Primary (a) and secondary (b) 18S rRNA nested-PCR electrophoresis images of *Cryptosporidium* detection from biofilm samples, using KLJ (outer) and CPB-DIAG (inner) primers. Visible bands appear in the second round at the ~435 bp.

Table 4.6.: Comparison Between IFA and Nested-PCR Detection of C*ryptosporidium* from Biofilms

Site Ref. (Region)	Sample ID	IFA	Nested-PCR
Site C (Black River Capital)	C1Bio	-	-
	C2Bio	+	+
	C3Bio	+	-
	C4Bio	-	-
Site D (Lacovia)	D1Bio	-	+
	D2Bio	-	-
	D3Bio	-	+
	D4Bio	+	+
Site E (Bartons)	E1Bio	-	-
	E2Bio	-	-
	E3Bio	-	-
	E4Bio	+	-
Total	**-**	**4**	**4**

4.4 Detection of *Cryptosporidium* from Cattle

4.4.1 Comparative evaluation of MAF, ELISA and PCR techniques

One hundred and nineteen fecal specimens were collected from 59 beef cattle and 60 dairy cattle, from 10 farms in February, March and October 2017. Because multiple techniques were employed for *Cryptosporidium* detection, the data was interpreted both collectively (based all three methods) and separately (based on individual method). In addition, diagnostic agreement between MAF microscopy and ELISA antigen test was evaluated as per Byrt (1996)'s classification scheme, previously summarized in Table 3.1.

Through MAF staining, oocysts appeared a deep pink colour, with a size ranging between 4-6

µm in diameter (Figure 4.8). For the ELISA antigen test, positive wells, which previously contained incubated specimens appeared yellow with an absorbance reading ≥0.150 at 450nm. Results are summarized in Table 4.7. according to cattle age group, production type, and diarrheic symptoms. Complete data is available in Table C.II., Appendix C.

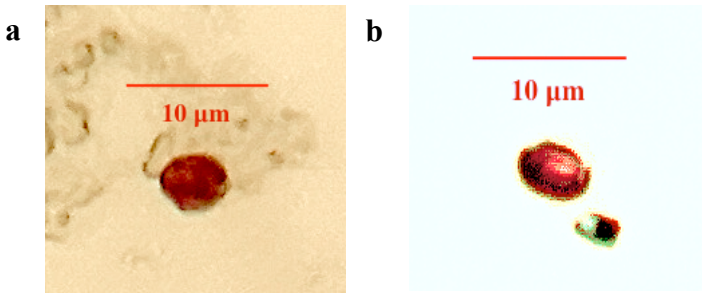

Figure 4.8.: *Cryptosporidium* oocysts detection from cattle sample 56DaCw (a) and positive control (b) by PBS-ether sedimentation and MAF staining. The Ward's® Chemistry Acid-Fast Stain Kit was used to stain oocysts, as per the CDC modified acid-fast staining procedure, 100X with immersion oil.

'Total' column displayed in Table 4.7. shows collective results, indicating that *Cryptosporidium* was detected in 39 (33%) samples by any one or more technique. Meaning that, if a single method confirmed a positive detection, while the other methods were negative, the sample was deemed positive. When analyzing the data separately, based on individual method, MAF-staining microscopy shows highest positivity with 29 (24%), followed by ELISA antigen test with 24 (20%), and conventional PCR with 10 (12%). Diagnostic agreement between MAF microscopy and ELISA antigen test, showed a *slight* (k= 0.39) agreement beyond chance.

Table 4.7.: *Cryptosporidium*-positive Detections by MAF, ELISA and PCR in Relation to Age, Production Type, and Diarrheic Symptom

Age/Production Type/Diarrheic Symptoms	No. of Samples Analyzed	No. of *Cryptosporidium*-positive Detections							
		MAF	Freq. (%)	ELISA	Freq. (%)	PCR	Freq. (%)	Total[b]	Freq. (%)
0 to 0.5 yr	23	5	22	5	22	2	9	6	26
0.6 to 1 yr	26	6	23	6	23	3	12	7	27
2 to 3 yrs	37	9	24	6	16	3	8	12	32
4 to 5 yrs	17	5	29	5	29	2	12	9	53
≥6 yrs	16	4	25	2	13	0	0	5	31
Dairy	60	18	30	17	28	7	12	25	42
Beef	59	11	19	7	12	3	5	14	24
Diarrheic (moderate or severe)	39	11	28	10	26	7	18	13	33
Non-diarrheic	80	18	23	14	18	3	4	26	33
Total[a]	**119**	**29**	**24**	**24**	**20**	**10**	**12**	**39**	**33**

[a] sum of all cattle specimens analyzed
[b] sum of *Cryptosporidium*-positive detection by any one or more method

4.4.2. Age-related, Production Type, and Diarrheic Symptom

The prevalence of *Cryptosporidium* based on cattle age, demonstrates highest prevalence at age 4 to 5 years. Despite this observation, the differences in detection by individual method were not statistically significant ($p > 0.05$). Therefore, there were no age-related correlation between positive detections. Notably, there were no PCR detections from cattle >6 years of age.

The prevalence of *Cryptosporidium* detected in beef cattle compared to dairy cattle is shown in

Figure 4.9. *Cryptosporidium* was confirmed in 14 (24%) beef, compared to 25 (42%) dairy by any one or more method. Collectively, positive detections were significantly higher in dairy cattle (p=0.04) compared to beef. When analyzing the data separately, 9, 12, and 5% of beef cattle, compared to 30, 28, and 12% of dairy cattle were positive by MAF, ELISA, and PCR, respectively. Statistical analysis determined that ELISA positives in dairy cattle were more prevalent than in beef cattle with significant correlation (p=0.02); conversely, MAF positives in dairy cattle showed no correlation (p=0.14).

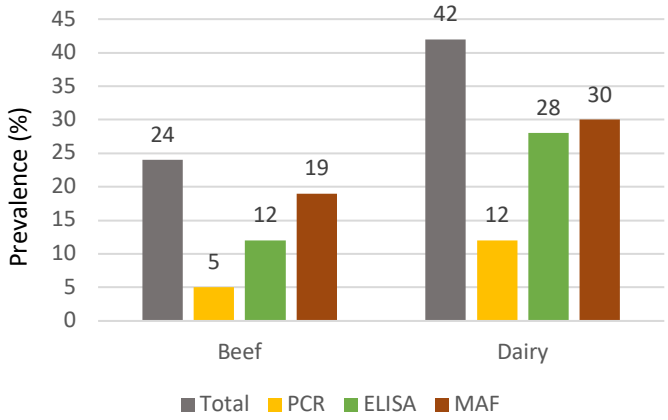

Figure 4.9.: *Cryptosporidium* detection by three different techniques in dairy and beef cattle. Total column refers to the number of positive detections by any one or more technique.

With regards to diarrheal symptoms, *Cryptosporidium* was detected by any one or more method from 13 of 39 (33%) diarrheic cattle (experiencing moderate or severe symptoms) and 26 of 80 (33%) non-diarrheic cattle. Therefore, both symptomatic and asymptomatic animals were carriers of the infection, showing no significant correlation with positive detections (p = 0.71). Notably, 7 of 10 (70%) PCR-positive detections were from cattle that had moderate or serve diarrhea at the time of sample collection. Therefore, most molecular detection of *Cryptosporidium* was from cattle experiencing moderate-to-severe diarrhea.

4.3.3. Farm and Management

Cryptosporidium detection per farm is presented in Figure 4.10. All farms except for F2 were positive for *Cryptosporidium* by any one or more method, with prevalence ranging from 10-56%. It is important to note that only 5 fecal specimens were collected from F2. Farms F4 and F7 show highest positivity, with 10 (50%) and 10 (56%) cows infected by any one or more method, respectively.

In terms of PCR detections, it was determined that *Cryptosporidium* was present on 8 of 10 farms. Positive detection by PCR was not obtained from any of the 5 or 7 cattle that were housed on F2 and F5, respectively. Electrophoresis images of PCR-positive detections are provided in Figure D.I., Appendix D.

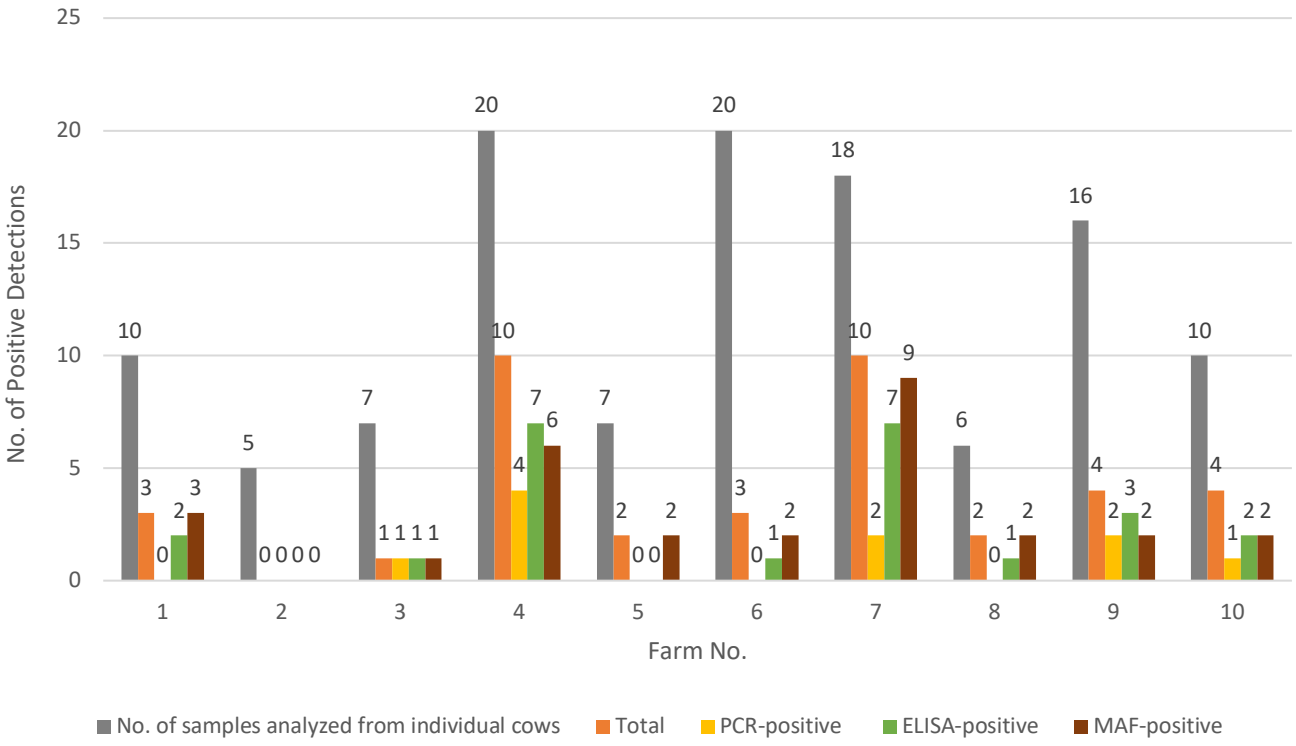

Figure 4.10.: *Cryptosporidium* detection by three different techniques in relation to farm. Total column refers to the number of positive detections by any one or more technique.

With regards to farm management, an informal assessment was conveyed. Cattle on all ten farms were raised outdoors; either in a restricted space or freely roaming on grassland with large fenced enclosures. All cattle were potentially exposed to dogs, rodents and other wildlife animals. Farms consisting of beef cattle roamed the pasture freely or were spaced out. Dairy cattle were confined to small spaces to facilitate grain feeding and milking; thus, were exposed to each other's feces. Typically, pre-weaned calves on dairy farms were raised on concrete that was sheltered by zinc roofing. Unlike beef farms, it was common practice for dairy adult cattle to be separated from calves.

4.4. Molecular Characterization and Phylogenetic Analysis of *Cryptosporidium spp.* and Subtypes

4.4.1. 18S rRNA Sequence Analysis and Identified Species/Genotypes

Fourteen samples derived from biofilm (n=4) and cattle (n=10) sources were determined positive for *Cryptosporidium* by either conventional or nested-PCR. Positive detections were subjected to genotyping through sequence analysis of the 18S rRNA gene. Clustal X alignment and NCBI BLAST confirmed 8 nucleotide sequences, which matched pre-existing genotypes in the GenBank database. The remaining 6 sequences had no match, presumably due to low quality of DNA and/or very low DNA targets present in the samples.

Genotyping of Biofilm Samples. Four biofilm samples, C2Bio, D1Bio, D3Bio, and D4Bio were confirmed positive by nested-PCR (Table 4.8.). Sequence analysis was successful and showed that all 4 positive detections had ≥ 98% homology with *C. parvum* (accession number: MF074701.1). This accession number matched a *C. parvum* genotype, which was previously identified in pre-weaned dairy calves in Shanghai China (Cai et al., 2017).

Table 4.8.: Identified *Cryptosporidium sp.* from Biofilm Samples by Partial Sequencing of the 18S rRNA Gene

Site (Region)	Sample I.D.	Species	Identity %	GenBank Accession No.
Site C (Black River Capital)	C2Bio	*C. parvum*	98	MF074701.1
Site D (Lacovia)	D1Bio	*C. parvum*	98	MF074701.1
	D3Bio	*C. parvum*	99	MF074701.1
	D4Bio	*C. parvum*	98	MF074701.1

Genotyping of Cattle Samples. Ten cattle specimens were determined positive by conventional PCR, as displayed in the electrophoresis images of Figure D.I., Appendix D. DNA of these samples were also subjected to nested-PCR; however, only 4 were confirmed positive. DNA from samples—within ±50 bp of the 435 bp target—were also subjected to nested-PCR; however, no positives were observed. Electrophoresis imaging of the primary and secondary PCR products are provided in Figure D.II., Appendix D. In the primary round, samples C34Cw, C53Cw, 105Cw were found positive as revealed by amplification at the 1325 bp. Primary PCR products were employed as templates in the secondary round, and visible bands appeared at the ~835 bp for C13Cw, C34Cw, C53Cw, 105Cw. Second-round amplification was also observed in the positive control, while no amplification was observed in the negative control.

Additionally, the concentration of DNA in each sample was evaluated using the intensity ranking scheme developed by Adamska et al. (2012). The intensity rankings obtained by conventional and nested-PCR are shown in Table 4.9. The intensity of DNA bands obtained with nested-PCR was either very strong (++++) or completely absent (-). Whereas, the intensity rankings obtained with conventional PCR varied: 3 were very strong, 1 was strong (+++), 3 were moderate (++),

and 3 were weak. Interestingly, nested-PCR using Xiao primers was more sensitive for higher concentration of DNA. However, for low DNA present in the cattle samples (~10 ng/µl), conventional PCR using CPB-DIAG primers was more sensitive. Given these results, only conventional PCR products were sequenced.

Table 4.9.: Intensity Rankings of Electrophoresis Bands, Comparing Conventional and Nested PCR-positive Detections from Cattle

Sample I.D.	Results of Conventional PCR	Results of Secondary Nested-PCR
C8Cw	+++	-
C13Cw	++	++++
34DaCw	++++	++++
40DaCw	+	++++
46BeCw	++	-
53DaCw	++++	++++
56DaCw	+	-
64BeCw	++	-
78Cw	+	-
105Cw	++++	-

++++ = very strong intensity of the DNA band
+++ = strong intensity of the DNA band
++ = moderate intensity of the DNA band
+ = weak intensity of the DNA band
– = no visualization of the DNA band

As shown in Table 4.10., 18S rRNA sequencing identified *C. parvum* and *C. hominis* species from 4 of 10 positive cattle samples. The number of species and corresponding GenBank accession numbers are as follows: *C. parvum* was identified in 3 samples: 34DaCw (accession number: AB513881.1), 53DaCw (accession number: MF074701.1), and 105Cw (accession number: MF074701.1); *C. hominis* was identified in 1 sample: C8Cw (accession number:

KX342865.1).

C. parvum genotype detected in dairy cow 34DaCw, matched with 99% homology to a sequence identified from dairy calves (AB513881.1) in Egypt (Amer et al., 2010). Similarly, *C. parvum* genotype detected in dairy cattle 105Cw and 53DaCw, matched with 99% homology to the same isolate identified in all biofilm samples, which was identical to that of dairy calves from Shanghai, China (Cai et al., 2017). *C. hominis* genotype (KX342865.1) detected in beef cow C8Cw was a direct submission to the GenBank database. The isolate matched with 78% homology to a genotype identified in human patients from New Delhi, India. Notably, all *Cryptosporidium spp*. were identified from cows that were ≤ 2 years of age, including two pre-weaned calves; C8Cw and 53DaCw.

Table 4.10.: Identified *Cryptosporidium spp.* and Genotypes from Cattle by Partial Sequencing of the 18S rRNA Gene

Farm I.D.	Sample I.D.	Species	Identity %	GenBank Accession No.
F3	105Cw	*C. parvum*	88[a]	MF074701.1
F4	C8Cw	*C. hominis*	78[a]	KX342865.1
	C13Cw	-	-	-
	40DaCw	-	-	-
	46BeCw	-	-	-
F7	53DaCw	*C. parvum*	99	MF074701.1
	56DaCw	-	-	-
F9	34DaCw	*C. parvum*	99	AB513881.1
	78Cw	-	-	-
F10	64BeCw	-	-	-

[a] unreliable identified *Cryptosporidium spp.* due to low % identity

Sequence analysis of the 18S rRNA was also performed on PCR products that showed non-specific bands. As previously mentioned, non-specific binding is a limitation of PCR assays, regarding *Cryptosporidium* detection from environmental samples (section 1.1.6.). This makes it difficult to assert representation of false-negatives. For example, of the 12 biofilm and 119 cattle samples, 8 non-specific bands were observed at the ~485 bp, which is slightly higher than the targeted 435 bp. These samples include: E2Bio, C9Cw, C12Cw, C18Cw, C20Cw, C22Cw, C24Cw and 22BeCw (refer to Figure D.I., Appendix D). Evidently, sequence analysis did not identify any *Cryptosporidium spp.* from these samples; although, Herbinix and Chania multitudinisentens bacteria were identified. Herbinix *sp.* are cellulose degrading bacteria (Koeck et al., 2015), while Chania multitudinisentens are bacteria found in soil landfill sites (Ee et al., 2016), which explains why they were consumed by cattle.

4.4.2. Phylogenetic Analysis of 18S rRNA Sequences

Phylogenetic analysis confirming *Cryptosporidium spp.* and genotypes is provided in Figure 4.11. Multiple sequence alignment was performed on *C. parvum* (MF074701.1) and *C. hominis* (KX342865.1) genotypes, which were used for constructing the phylogenetic tree. The Neighbour-Joining method grouped all *C. parvum* isolates from this research with the designated reference species in 1 cluster (97% bootstrap support). Similarly, the *C. hominis* isolate was grouped with the reference species in 1 cluster (64% bootstrap support). Multiple aligned sequences are provided in Figure 4.12., highlighting the variable region, which distinguishes *C. parvum* and *C. hominis*. Chromatogram readings of the aligned sequences, showing good quality are provided in Figure E.I., Appendix E.

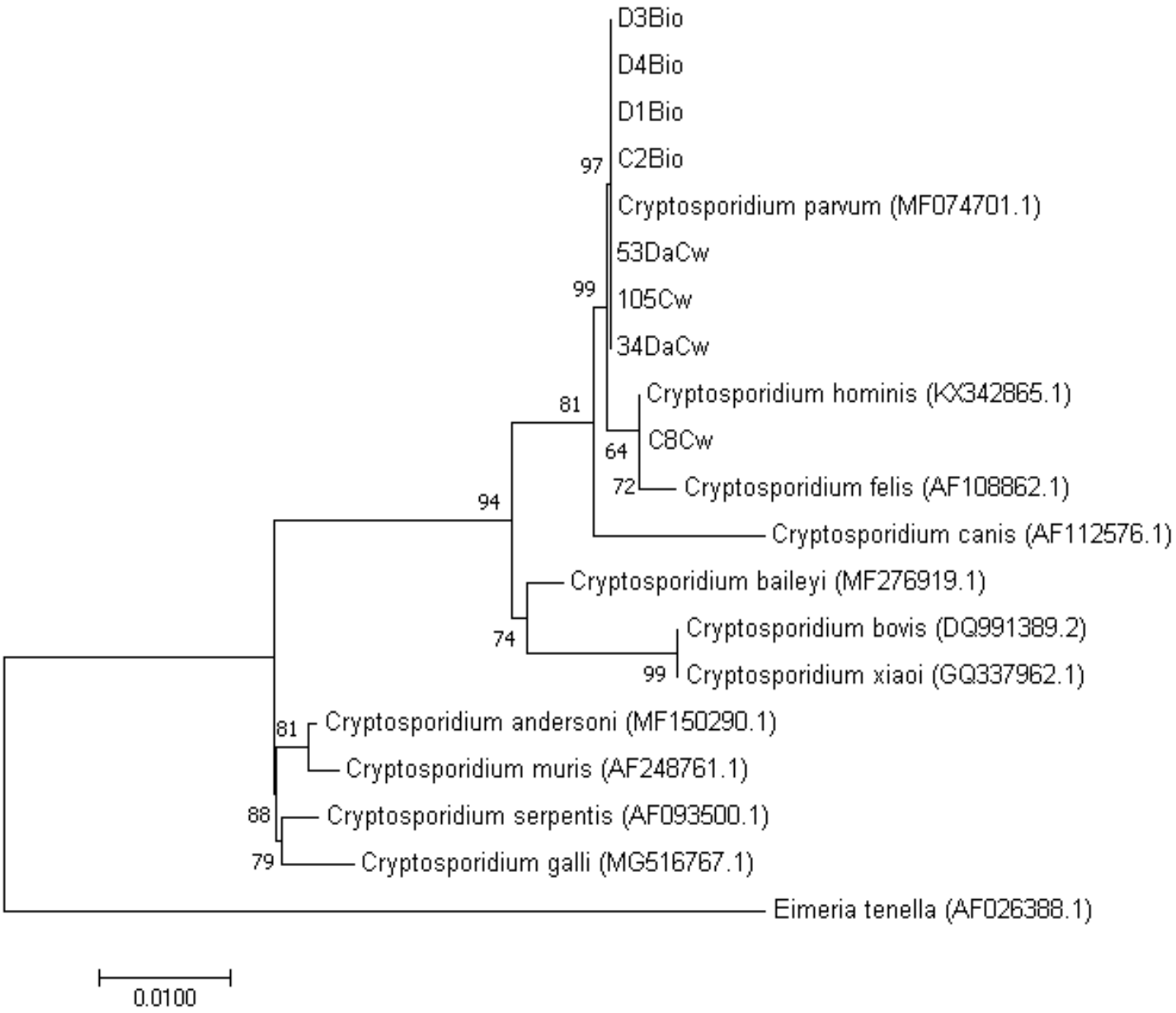

Figure 4.11. **Phylogenetic relationships of *Cryptosporidium* isolates from cattle inferred by Neighbor-Joining analysis of 18S rDNA sequences in MEGA7.** Reference species: *C. parvum* (MF074701.1) and *C. hominis* (KX342865.1). Outgroup species: Eimeria tenella (AF026388).

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C8Cw          TTTTAGWATATGAAATTTTACTTTGARAAAATTARAGKGCTTAARGCAGGCWTWTGCCTT     60
105Cw         TTTTAGWAWATRAAATTTTACTTTRARAAAATTARAGKGCTTAAAGCAGGCATATGCCTT     60
D3Bio         TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
34DaCw        TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
53DaCw        TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
D4Bio         TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
D1Bio         TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
C2Bio         TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
C.parvum      TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
C.hominis     TTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTT     60
              ****** * ** ************ * ******* ** ****** ****** * ******

C8Cw          GAAMMCYCCAGCAKGRAAWAAWATWAAARATTTTTWTYTTTTTTWTKGGTTCTAARAWAA    120
105Cw         GAAWACYCCASCAKGRAAWAAWATWAAARATTTTTATCTTTCTWATKGGTTCTAARATAA    120
D3Bio         GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
34DaCw        GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
53DaCw        GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
D4Bio         GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
D1Bio         GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
C2Bio         GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
C.parvum      GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAA    120
C.hominis     GAATACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTTTTATTGGTTCTAAGATAA    120
              ***  * *** ** * ** ** ** *** ****** * *** *  * ******** * **

C8Cw          RAAWAAKGWTTAAWAGGGACRGTKGGGGGCWTTTKTWTTTAACAGTCRRAGGKGAAWTTY    180
105Cw         RAATAAKGATWAAWAGGGACAGTKGGGGGCWTTTGTATTTAACAGTCARAGGTGAAWTTC    180
D3Bio         RAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
34DaCw        RAATAATGATTAATAGGGACAGTTGGGGGCWTTTGTATTTAACAGTCAGAGGTGAAATTC    180
53DaCw        RAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
D4Bio         RAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
D1Bio         RAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
C2Bio         GAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
C.parvum      GAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
C.hominis     GAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTC    180
               ** ** * * ** ****** ** ****** *** * **********  *** *** **

C8Cw          TTARWTTTGTTAAARACAAAYTAWKGCRAAAGCWTTTGCCARGWWTKTTTTCWTTAWTCA    240
105Cw         TWARATTTGTTAAARACAAACTAAKGCRAAAGCWTTTGCCAAGGATGTTTTCWTTAATCA    240
D3Bio         TTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCA    240
34DaCw        TTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCA    240
53DaCw        TTAKATTTGTTAAAGACAAACTAATGCGAAAGCWTTTGCCAAGGATGTTTTCATTAATCA    240
D4Bio         TTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCA    240
D1Bio         TTAGATTTGTTAAAGACAAACTAATGCGAAAGCWTTTGCCAAGGATGTTTTCATTAATCA    240
C2Bio         TTAGATTTGTTAAAGACAAACTAATGCGAAAGCWTTTGCCAAGGATGTTTTCATTAATCA    240
C.parvum      TTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCA    240
C.hominis     TTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCA    240
              * *  ********* ***** **  ** ***** ******* *  * ***** *** ***

C8Cw          ARAACRAARGTRRGGGGAMCRAARACRAMCARATMCCGYCGWAKYCTTAACCAWAAACTA    300
105Cw         ARAACRAAAGTTAGGGGATCGAARACRATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
D3Bio         AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
34DaCw        AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
53DaCw        AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
D4Bio         AGAACGAARGTTAGGGGATCGAARACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
D1Bio         AGAACGAARGTTAGGGGATCGAAGACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
C2Bio         AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAKTCTTAACCATAAACTA    300
C.parvum      AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTA    300
C.hominis     AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTA    300
              * *** ** **  ***** * ** ** * ** ** *** ** *  ******** ******
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C8Cw            TGCCAACTARARATKGG        317
105Cw           TGCCAACTAGAGATTGG        317
D3Bio           TGCCAACTAGAGATTGG        317
34DaCw          TGCCAACTAGAGATTGG        317
53DaCw          TGCCAACTAGAGATTGG        317
D4Bio           TGCCAACTAGAGATTGG        317
D1Bio           TGCCAACTAGAGATTGG        317
C2Bio           TGCCAACTAGAGATTGG        317
C.parvum        TGCCAACTAGAGATTGG        317
C.hominis       TGCCAACTAGAGATTGG        317
                ********* * ** **
```

Figure 4.12.: Clustal X aligned nucleotide sequences obtained from PCR-positive biofilm and cattle samples, reference species: *C. parvum* (MF074701.1) and *C. hominis* KX342865.1). Residues conserved in all species are shown (asterisks) and variable region highlighted (blue box).

4.4.3. Gp60 Sequence Analysis and Identified Subtype

Gp60 analysis to identify *Cryptosporidium* subtypes was important because phylogenetic analysis of *C. parvum* and *C. hominis* at the 18S rRNA (hypervariable region) has shown to generate a similarity of 97.7% (Morgan, Monis, Fayer, Deplazes, & Thompson, 1999). Likewise, the percent identity of sequences from this study has shown a similarity of 99.68% amoung *C. parvum* and *C. hominis* detections (Figure 4.13.). Therefore, the two species are nearly identical at the 18S rRNA locus, which could lead to misinterpretation.

```
 1: C8Cw         100.00   84.86   77.92   78.23   78.23   78.55   78.55   77.92   77.29   77.60
 2: 105Cw         84.86  100.00   88.33   88.64   88.64   88.33   88.33   88.33   87.70   87.38
 3: D3Bio         77.92   88.33  100.00   99.68   99.37   99.37   99.37   99.37   99.37   99.05
 4: 34DaCw        78.23   88.64   99.68  100.00   99.05   99.05   99.05   99.05   99.05   98.74
 5: 53DaCw        78.23   88.64   99.37   99.05  100.00   98.74   99.37   99.37   98.74   98.42
 6: D4Bio         78.55   88.33   99.37   99.05   98.74  100.00   99.37   98.74   98.74   98.42
 7: D1Bio         78.55   88.33   99.37   99.05   99.37   99.37  100.00   99.37   98.74   98.42
 8: C2Bio         77.92   88.33   99.37   99.05   99.37   98.74   99.37  100.00   99.37   99.05
 9: C.parvum      77.29   87.70   99.37   99.05   98.74   98.74   98.74   99.37  100.00   99.68
10: C.hominis     77.60   87.38   99.05   98.74   98.42   98.42   98.42   99.05   99.68  100.00
```

Figure 4.13.: Percent Identity Matrix created by Clustal2.1, showing relationship between identified sequences with reference species.

gp60 PCR			gp60 Seq.
Well no.	**Sample I.D.**	**Result**	**Result**
1,2	C8Cw	-	N/A
3.4	C13Cw	-	N/A
5,6	34DaCw	-	N/A
7,8	40DaCw	-	N/A
9,10	46BeCw	-	N/A
11,12	53DaCw	-	N/A
13, 14	56DaCw	-	N/A
15,16	64BeCw	-	N/A
17, 18	105Cw	+	**IbA9G2**
19, 20	78Cw	-	N/A
21, 22	C2Bio	-	N/A
23, 24	D1Bio	-	N/A
25, 26	D3Bio	-	N/A
27, 28	D4Bio	-	N/A
29	**PC**	+	**N/A**
30	NC	-	N/A
31	NC	-	N/A

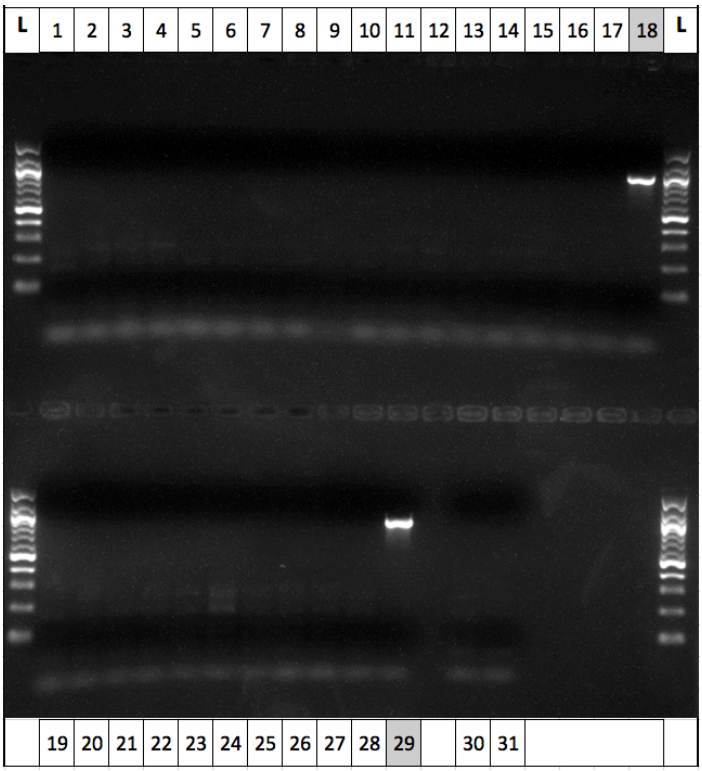


Figure 4.14.: Secondary Nested-PCR electrophoresis image of *Cryptosporidium* detection from cattle samples at the gp60 locus and corresponding subtype identification. PC is the positive control.

Here, gp60 analysis was performed by sequencing the highly polymorphic locus, to identify specific subtypes of *Cryptosporidium*-positive detections. In addition, gp60 analysis was performed to assess whether the subtypes are globally widespread, as well as known to cause zoonosis and/or severe symptoms in human populations. DNA from all 14 PCR-positive biofilm and cattle specimens were subjected to a nested-PCR that amplified a fragment of the gp60 gene. Figure 4.14. shows the electrophoresis imaging of the PCR products in the nested round. Visible bands only appear at a single sample, 105Cw, as well as the positive control. Sequence analysis identified *C. hominis* subtype IbA9G2, corresponding with GenBank accession number AY166807 with 99% homology. Previous studies have identified this particular subtype in humans (Pelayo et al., 2015), cattle (Razakandrainibe et al., 2018) and river water (Gertler et al.,

2015). It is important to note that the *C. hominis* subtype identified from sample 105Cw contradicts the previous identification of *C. parvum* at the 18S rRNA locus. It is widely accepted that sequencing the gp60 gene, including the microsatellite region is a more specific target compared to the hypervariable 18s rRNA region. Thus, gp60 sequencing produces higher resolution data compared to 18S rRNA, which often overlays different sequences. Moreover, 18S rRNA sequencing of 105Cw corresponded to *C. parvum* with 88% homology; while gp60 sequencing of 105Cw corresponded to C. *hominis* with 99% homology. For this reason, 105Cw was believed to be C. *hominis* as determined through gp60 analysis.

Chapter 5: Discussion

5.1. Main Findings

5.1.1. Assessment of in situ biofilm roughness and thickness for Cryptosporidium detection

In situ biofilm development was shown to be effective for *Cryptosporidium* detection in surface water. However, based on available literature, it is clear that the approach's success depends vastly on the ability for biofilms to obtain optimal structure that is favourable for oocysts capture and retention. Findings from the pilot study reveal that all biofilms reached a heightened stage of maturation suitable for oocysts entrapment, as determined by average thickness and maximum average thickness algorithms in COMSTAT2.

Here, biofilms attained an average thickness of 48.33 to 82.53 μm, with a maximum average thickness of 95.63 to 115.13 μm following 3-weeks of incubation in creek water. These results are comparable to previous *Cryptosporidium*-biofilm investigations. Wolyniak et al. (2010) performed an *in vitro* study that portrayed a realistic depiction of oocysts entrapment in environmental biofilms collected from stream rocks. The biofilms were inoculated into a flow-cell system, using *C. parvum* oocysts and filtered creek water as the flow-through medium. After eight days, the biofilms reached an average thickness between 25 and 42 μm, which showed to capture and sustain oocysts for at least 25 days. These average thickness measurements were slightly lower than determined in the present study; however, the biofilms were incubated for a shorter length of time.

Koh et al. (2013) also reported significant increases in oocysts recovery ($p< 0.001$) as biofilm thickness increased from 21 to 105 μm. It is important to disclose that even immature biofilms as thin as 0.7 μm (Koh et al., 2013) and 2.81 μm (Searcy et al., 2006) have shown to capture

oocysts within a flow-cell system. Despite these conflicting observations, optimization of oocysts entrapment seemly occurs as biofilms mature to a thickness of approximately 100 μm (Koh et al., 2013), which was met by all biofilms from the pilot study.

Biofilms with 100 μm thickness fulfil the penultimate stage of development, in which cell clusters are nonmotile and have reached a plateau (Davies et al., 1999; Sauer, Camper, Ehrlich, Costerton, & Davies, 2002). At this stage, several layers of microorganisms have been added to the biofilms; thus, oocysts bury within denser regions, resulting in fewer detachments (Rogers & Keevil, 1995; Warnecke, 2006). Similarly, a shielding-effect is produced on account of biofilm roughness. According to Sendamangalam (2012), biofilm roughness generates voids, creating quiescent zones (areas where fluid flow is absent) for pathogens to inhabit. In the pilot study, the mean roughness coefficient was >1. This is reflective of voids, also described as biofilm patchiness or heterogeneous formation; having minimal growth on some areas of the substrate (Merod et al., 2007; Murga, Stewart, & Daly, 1994)

Overall, biofilm structure including thickness and surface roughness are critical factors to consider when monitoring *Cryptosporidium spp.* in environmental waters. Previous studies have shown that an increase in biofilm maturation correlates with oocysts embedment. However, it is important to note that excessive biofilm formation can result in sloughing; the dispersal of cell clusters from the interior portion of the biofilm. This process is completely randomized, often depending on the linear-flow velocity of water (Angles, Chandy, Cox, Fisher, & Warnecke, 2007). Thus, it is unclear how widespread this phenomenon is amoung biofilms that have developed under different environmental conditions, let alone have diverse microbial composition. Nonetheless, studies have reported that sloughing occurs immediately after the 100 μm threshold (Sauer et al., 2002). In the present study, several biofilm segments reached a

thickness >115 µm after 3-weeks (Table C.V, Appendix C); thus, it is presumed that sloughing may have occurred during the sample collection period. Based on these observations, a limiting factor of the current *biofilm sampler* prototype is that it may not be appropriate for predicting prevalence nor comparing point-source loading of *Cryptosporidium* oocysts in a given water source, as attempted here and in previous investigations (e.g., Barnes-Pohjonen, 2012; McLeod, 2011; PWD, 2014). Although, this may be applicable with greater understanding biofilm development in natural waters and oocysts recovery efficiency. Nonetheless, the technique remains beneficial for monitoring the absence or presence of *Cryptosporidium,* as well as determining its species and genotypes from environmental waters, which is discussed further in section 5.1.6.

5.1.2. Biofilm sampling generates comparable oocysts recovery efficiencies to standardized filtration

Biofilm sampling was shown to generate oocysts recovery rates that were comparable to Method 1623, and in some cases, more efficient. It is important to acknowledge that the analysis of seeded oocysts from biofilms followed the same IMS-IFA procedure as Method 1623. The only difference was that REF coverslips were used to facilitate scanning through slides for oocysts quantification (section 3.1.5.).

Mean oocysts recovery from biofilms was 42.9% ± 18.45 and ranged between 23.9 and 68.7%. These results are similar to Wolyniak et al. (2010), which determined oocysts recoveries from environmental biofilms between 24 and 65%. Furthermore, a large-scale study recovered seeded oocysts from various water sources through filtration, using the same Dynabeads™ anti-*Cryptosporidium* kit. The study determined a mean oocysts recovery of 48.4% ± 11.8 from tap

water; while from raw water, the recovery percentages fluctuated between 19.5 to 54.5% (McCuin and Clancy, 2003). Several other studies have confirmed fluctuating oocysts recoveries, between 1 and 61%, from non-finished water sources including raw water, backwash and wastewater (Di Giovanni et al., 1999; Ferrari, Stoner & Bergquist, 2006). Evidently, oocysts recovery by means of biofilm sampling is similar to that of environmental and wastewater filtration methods because they produce inconsistent results.

Inconsistent recovery rates from environmental sources are caused by several factors. Low efficiencies are reportedly due to significant losses of oocysts during the filtration step (Feng et al., 2003; Francy et al., 2004; Hu et al., 2004). In the present study, the biofilm sampling process eliminates the filtration step entirely; thus, the number of oocysts overlooked was potentially reduced. Low IMS recovery is also generated due to the presence of inhibitors. The presence of divalent cations and suspended solids in environmental and wastewaters influence the binding capacity of the antibody-coated IMS beads to oocysts (WHO, 2009). Ageing of oocysts also strips the epitopes from the outer shell of oocysts, further preventing antibody attachment. In such cases, IMS recovery from biofilms may have been disadvantageous because extensive attachment of natural organic matter (NOM) to oocysts has shown to result in steric repulsion (Dai & Hozalski, 2002). Perhaps this occurred at Site E, wherein extensive NOM accumulation could be observed at the location during the sampling period (Figure G.I, Appendix G). In effort to mitigate this repulsive effect, the pH of all biofilm suspensions was adjusted to a neutralized charge, which has previously shown to optimize bead-binding capacity in water containing inhibitors (Dai & Hozalski, 2002; Kuhn, Rock, & Oshima, 2002).

With regards to suspended solids, turbidity is also an important factor to consider when performing IMS recovery of oocysts from biofilms. In deionized water, oocyst recovery is

typically at an optimum range (between 76% to 83%). However, at very high turbidity levels (e.g., 5000 NTU), the recovery of oocysts from water is usually < 35%, depending on the kit used (Bukhari et al, 1998). In the present study, mean turbidity measured form the biofilm suspensions was 548.1 ± 4.73% NTU. Presumably, this level did not affect IMS because 500 NTU is said to yield optimal results (Feng et al, 2003). Notably, several studies have also suggested that turbidity has minimal effect on oocyst recovery via IMS (Rochelle et al, 1999). Overall, divalent cations and suspended solids including NOM, potentially affected the IMS step of Method 1623 causing variable recovery rates that were unique to each biofilm culture. Whether these results were due to pH, surface charge interactions, physical NOM barriers, or a combination of these factors needs to be investigated further.

5.1.3. Concentrated and pure DNA extracted from oocysts derived from biofilm samples

The isolation and enrichment of *Cryptosporidium* DNA, free of significant contaminates, is imperative for successful genotyping. According to Genomics Core Facility (n.d.), DNA concentration of purified PCR products should be at least 20 ng/µl, to produce quality sequences, especially when using an automated sequencer, as in the present study. Through spectrophotometric analysis, a DNA concentration of 319.11 ng/µl and 267.13 ng/µl was determined from the purified PCR products of biofilms C2Bio and D3Bio, respectively, indicating that *Cryptosporidium* genomic DNA was present in the biofilms at very high concentrations. With regards to purity, the A_{260}/A_{280} and A_{260}/A_{230} ratios of sample C2Bio and D2Bio were 2.27 and 2.24, respectively. As previously mentioned, the recommended "pure" range is typically between 1.8 and 2.2 (Thermo Scientific, 2009); thus, DNA extracted from these biofilm sources were very pure.

A possible explanation for such abundant and pure DNA is that the outer shell of several oocysts remained intact while embedded within biofilms. This potentially aided in the maintenance of DNA integrity. It is believed that because biofilms have polysaccharide extracellular matrices in which oocysts are embedded, they are also capable of providing temporary relief against adverse environmental conditions, as well as mitigating factors such as aging, which cause the cells to crack open resulting in the disturbance of internal DNA (Anguish & Ghiorse, 1997). Overall, these finding support the notion that biofilms serve as an active membrane continuously providing attachment and protection for pathogenic cells, including *Cryptosporidium*. Although, further research involving a larger sample size and oocysts viability is necessary to support this presumption.

5.1.4. Biofilm sampling as an alternative to conventional filtration

In the event of large outbreaks, analysis of multiple samples is necessary to provide spatial and temporal assessment within a given region. Arguably, the high cost associated with filtration and IMS-microscopy-based detection, impedes on effective monitoring. Typically, identification of *Cryptosporidium spp.* from environmental waters involves collecting single grab-samples through filtration, in accordance with EPA Method 1623, then analyzing the samples through some form of genotyping assay. While informative, the current approach is often compromised due to poor recoveries, and limited snapshot data at the time of sample collection. Additionally, most water-monitoring programs require Envirochek™ Regular or HV Sampling Capsules (Pall Corporation, Ann Arbor, MI), which are membrane filters that replace traditional string wound filters; thus, increase the capture and recovery efficiency of *Cryptosporidium* oocysts > 70% (Pall Corporation, 2017). These capsules however, currently cost $149.98 USD each (VWR, 2017), and since multiple samples are required to obtain a comprehensive representation of

Cryptosporidium spp. in natural waters (Hsu et al., 2002), the overall expenditure of the technique can be costly, especially for long-term investigations. To overcome these limitations, biofilm sampling has shown to yield a gradual representation of *Cryptosporidium spp.* passing through water columns over a given period of time, opposed to instantaneous grab-sampling. In addition, the technique has shown to be cost-effective compared to standardized filtration, as the apparatus itself is re-usable, and the only disposable expense is the polymeric material used to create the slides (< $1 per slide) (refer to Figure F.I. in Appendix F).

In addition to cost, biofilm sampling generates comparable recovery efficiencies to standardized filtration because it concentrates oocysts in a similar manner to macroinvertebrates and filter-feeding sentinels. For example, researchers are constantly investigating alternate sources opposed to water filtrate for monitoring *Cryptosporidium spp.* in aquatic environments. Reboredo-Fernández et al. (2014) homogenized benthic macroinvertebrates including nymphs, water bugs and larvae from 9 rivers in Galicia, Spain and found *Cryptosporidium* oocysts in 12.5% of the samples via IFA-microscopy. Likewise, shellfish have become widely accepted as bioindicators of fecal contaminants, including bacteria, viruses and parasites (Potasman, Paz, & Odeh, 2002). Numerous studies have shown that shellfish including oysters, mussels, clams, and cockles, share the ability of bioaccumulating *Cryptosporidium* oocysts, while retaining them in their hemolymph, digestive glands, gastrointestinal tracts and on gills (Graczyk, Fayer, Lewis, Trout, & Farley, 1999; Guiguet Leal et al; 2008; Miller et al., 2005; Staggs et al., 2015). In many cases, *Cryptosporidium spp*. have been detected from shellfish, even when they were undetected from surrounding waters through filtration (as cited in Miller et al., 2005). Moreover, various *Cryptosporidium spp*. have been identified from shellfish through PCR and gene sequencing applications, providing epidemiological context to point-source transmission, as well as genotypes that are endemic in human populations (Gomez-Bautista et al., 2000; Miller et al.,

2006; Staggs et al., 2015). Work presented in this dissertation therefore, demonstrates that the same research outcomes can be achieved through biofilm sampling. Even further applications may be achieved, given the widespread prevalence of biofilms in various aquatic environment; whereas shellfish are limited to ambient waters.

5.1.5. High prevalence of Cryptosporidium infections in dairy cattle with potential association to rearing system

Cryptosporidium has been found in many livestock animals worldwide; however, infections are reportedly endemic in cattle species (Santín & Trout, 2008). In the present study, *Cryptosporidium* was detected in 14 (24%) beef cattle compared to 25 (42%) dairy cattle. These findings were based on the combined results of three different detection methods, which revealed significant prevalence in dairy cattle ($p=0.04$). This pattern was also observed separately by MAF, ELISA, and PCR methods, as 9, 12, and 5% of beef cattle, compared to 30, 28, and 12% of dairy cattle were positive, respectively. However, it is important to note that based on statistical analysis, only ELISA positive detections in dairy cattle showed higher prevalence than in beef cattle with significance ($p=0.02$); conversely, MAF positives in dairy cattle showed an insignificant correlation ($p=0.14$). A possible explanation is the *slight* diagnostic agreement beyond chance between MAF microscopy and ELISA antigen test. This highlights the importance of using multiple methods for detecting *Cryptosporidium* from cattle feces, especially since oocysts concentrations can be low and inhibitors are often abundant (i.e., cellulose inhibitors due to grass consumption).

Results demonstrating a high prevalence of *Cryptosporidium* infections amoung dairy cattle is supported by several epidemiological studies. Gong et al. (2017) determined that the rate of

infection in dairy cattle was more prevalent than in beef cattle (Gong et al., 2017). While another study based in the Canadian provinces of Alberta, Saskatchewan and British Columbia, found that mortality rates in beef calves increased by 30%, usually when dairy calves were introduced to the beef herds during calving season (Olson, Ralston, O'Handley, Guselle, & Appelbee, 2003). Moreover, a large-scale study, which identified *C. parvum* in 1253 calves from Czech Republic, determined that pre-weaned dairy calves had highest prevalence of infection up to 56.5% per farm; while only three cases of *C. parvum* oocysts shedding was found in pre-weaned calves from beef farms (Kváč, Kouba, & Vítovec, 2006). Therefore, previous studies support the observation that dairy cattle have a higher prevalence of *Cryptosporidium* infection than beef cattle.

The disparities between *Cryptosporidium* infection in dairy versus beef cattle may be attributed to differences in rearing systems. In the present study, an informal assessment of farms revealed that beef cattle typically roamed the pasture more freely or were spaced out. Whereas, dairy cattle were confined to small spaces to facilitate grain feeding and milking; thus, were exposed to each other's feces. Garro, Morici, Utgés, Tomazic, and Schnittger (2016) investigated the occurrences of *Cryptosporidium* in dairy calves from Argentina and suggested that this phenomenon is in part due to conditions of artificial rearing systems. Because dairy cattle are commonly raised in confinement, they are predisposed to rapid infection and subsequent re-infection. On the other hand, beef cattle are commonly kept outside in open ranges, which spreads the infection relatively slower. These claims are further supported by McAllister, Olson, Fletch, Wetzstein, and Entz (2005), which investigated the prevalence of *Cryptosporidium* detection in 49 dairy and beef farms across Ontario and British Columbia, Canada. The study observed that dairy calves allowed outside confinement were reportedly less likely to be infected by *Cryptosporidium* compared to their counterparts kept in barns. The differences in the rearing

and production systems accounted for the 63.3% infection rate in dairy cattle compared to the 18.4 % in beef cattle. Therefore, preventative measures pertaining to housing are crucial for mitigating the spread of cryptosporidiosis within dairy herds.

Adequate housing of cattle involves several strategies. Cattle should be kept in hygienic and spacious environments to prevent the risk of spreading the disease. Animal feeds and water are prone to contamination by oocysts; therefore, regular cleaning of pen floors and troughs, while minimizing contact between animals may aid in reducing spread. It is also essential to separate the feed from defecation areas. In the present study, most dairy farms had regular cleaning routines; although it appeared challenging for farmers to keep the feed separated from feces given the overcrowded feed-lots. Thus, beside cleaning, farms should mitigate overcrowding, as it has shown to increase animal contact, while presenting the challenge of upkeeping sanitary measures (Ghazy, Abel-Shafy, & Shaapan, 2016). Furthermore, sanitary or hygienic practices is most important for young dairy calves, which are more susceptible to the cryptosporidiosis compared to older animals. It is recommended to use concrete flooring for young calves because it makes cleaning easier, which was observed to be a common practice on most of dairy farms in the present study.

Overall, despite being discovered over 100 years ago, *Cryptosporidium* continue to be amoung the most difficult pathogens to control on cattle farms. As shown in the present study, *Cryptosporidium* was widespread on 9 of 10 farms, with highest prevalence in dairy cattle. Currently, there is no vaccine or drug available to combat bovine cryptosporidiosis, and our knowledge of host–pathogen interactions in cattle is very limited. Thus, future research should focus on understand prevalence and risk factors for oocysts shedding, to effectively monitor and implement control measures that can help mitigate the parasites' transmission within herds and

to the surrounding environment.

5.1.6. C. parvum and C. hominis isolates identified in biofilm and cattle samples

In the remote geographical region of St. Elizabeth, Jamaica, a low species diversity consisting of *C. hominis* and *C. parvum* genotypes was detected in biofilm and cattle samples. Generally, *C. hominis* is host-specific because it transmits primarily within human populations. On the other hand, *C. parvum* is considered anthropozoonotic because it transmits from both humans and animals including ruminants, domestic pets, and wildlife.

Identification of *Cryptosporidium sp.* from biofilm samples. *C. parvum* was the only species identified from the biofilm samples through 18S rRNA sequencing. These results are similar to that recently reported in Haiti; wherein, *C. parvum* was the sole species identified from 15 of 16 water samples collected from natural waters. The researchers suggested that the samples contained either a single species or multiple species that were undetected (Damiani et al., 2013).

If a single species was present in the biofilm samples, then there is a possibility that clonality contributed to low species diversity. Clonality effects the diversity of *Cryptosporidium spp.* because it causes a dominant mutant to exist within a population. This is true for species developed in isolation, which potentially occurred in the remote region of St. Elizabeth, Jamaica. For example, species that develop in isolation may experience genetic pool monotony, leaving the fate of a single, beneficial mutation to exist within a population. Thus, the interspecies competition may have lead to the extinction of diverse mutants (Ndaoa et al., 2013). Isolation also means that hosts are not exposed to different incoming species. Thus, sexual reproduction becomes an exchange of genetic material that is the same amoung members of the same species.

Previous studies have demonstrated that confinements in hosts have the same effects as confinement to geographic locations in relation to *Cryptosporidium* epidemiology (Wang et al., 2014).

Another explanation of low species diversity is that there were undetected species due to the use of basic molecular tools. In such cases, superimposition may have occurred during gene sequencing. For example, sequencing of the 18S rRNA does not account for species that co-exist within a single sample. In many cases, the sequences were unreadable due to the overlaying of different species, which can be observed from the chromatogram readings shown in Figure E.I., Appendix E. Also, a combination of clonality and superimposition may have occurred, where *C. parvum* was so prevalent that it masked the DNA from any other *Cryptosporidium spp.* co-existing in the same sample. Moreover, a single gene target was assessed at the 18S rRNA in this research, which does not account for other genes that may have been more prevalent.

Identification of *Cryptosporidium spp.* and subtype from cattle specimens. Four species of *Cryptosporidium* are typically found in cattle: *C. parvum, C. bovis, C. ryanae* and *C. andersoni* (Fayer, Santin, & Trout, 2008; Fayer, Santin, & Xiao, 2005). In the present study, *C. parvum* was identified in 2 cattle (≥ 98% homology); while *C. hominis* was identified in 2 cattle. It is important to note that one of the *C. hominis* isolates matched subtype IbA9G2 with 99% homology; while the other *C. hominis* isolate match with only 78% homology, indicating unreliable identification.

It was not surprising to have identified *C. parvum* from cattle because as previously mentioned, this species is most widespread amoung a diverse group of animals. According to Jamaica's Rural Agricultural Development Authority (RADA), St. Elizabeth is the leading livestock-producing parish in the country, having over 25,000 registered farmers (RADA, 2017). The

livestock rearing systems includes: goats, sheep, hogs, cattle and horses—all of which are known carriers of *C. parvum* (Fayer & Xiao, 2008). Thus, given the outdoor environment, in which cattle often cohabit with other livestock animals on the same agricultural land, the potential exposure of *C. parvum* was plausible. In contrast, it was surprising to have identified *C. hominis* from cattle because it is generally associated with human infections. Nonetheless, in low prevalence, earlier studies have identified *C. hominis* in cattle (through 18s rRNA sequencing) from Malawi (Banda, Nichols, Grimason, & Smith, 2009), Korea (Park et al., 2006), Scotland (Smith et al., 2005) and India (Feng et al., 2007).

Furthermore, the specific *C. hominis* IbA9G2 subtype was recently reported in symptomatic and asymptomatic calves in France (Razakandrainibe et al., 2018). This marked the first reported bovine case of gp60 *C. hominis* connected to the Ib subtype family. Interestingly, Gatei et al. (2008) also identified a *C. hominis* subtype from the Ib family in 71% HIV infected persons in Jamaica. The study confirmed that the subtype was geographically distinct to the Jamaican population, which potentially explains why this particular subtype family was found in cattle from the Black River watershed. Additionally, human cases of the IbA9G2 subtype was previously reported in humans from Australia (O'Brien, McInnes, & Ryan, 2008; Waldron, Ferrari, & Power, 2009), French Guiana (Mosnier et al., 2018), Europe (Gertler et al., 2015) and Cuba (Pelayo et al., 2008). Furthermore, Chalmers et al. (2008) conducted a case–control study and sequenced 115 isolates from the gp60 locus to further understand the geographical transmission of *C. hominis*. The study confirmed that subtype IbA9G2 was mainly linked to Europe because it was not connected to outside travel.

Finally, the IbA9G2 subtype was connected to a waterborne outbreak in Germany between May and June 2013, where an abnormal 24 cases of cryptosporidiosis occurred in the city of Halle.

The cases were investigated further, and only *C. hominis* of IbA9G2 subtype was detected from the patients' stool. The outbreak was attributed to extensive river flooding, which damaged sewage systems, resulting in an overflow into drinking water sources (Gertler et al., 2015). This study provides insight, potentially explaining how a *C. hominis* subtype was ingested by cattle in the Black River watershed. Presently, there are no existing sewage treatment plants in the parish of St. Elizabeth, Jamaica. The primary method of sewage disposal is hand-dug soak-away pits (Miller, Waite, & Harlan, 2001). Therefore, the absence of a centralized or formal sewage treatment system may have contributed to cattle exposure to human feces, particularly during times of heavy rain activity.

Overall, fecal contamination due to human effluent and livestock feces have led to several *Cryptosporidium* outbreaks in the past (Putignani & Menichella, 2010). The transmission of oocysts from humans and terrestrial animals to surface waters pose significant health risks to those utilizing the ecosystem. Although groundwater is the predominant drinking water source in St. Elizabeth, the Black River watershed is also used regularly for crop irrigation, recreational swimming and tourism. Thus, the presence of *C. parvum* and *C. hominis* in surface water and cattle feces suggests that both agricultural and human wastewater are potential sources contributing to *Cryptosporidium* infections in St. Elizabeth. However, additional work investigating human cases of cryptosporidiosis in the region is needed to draw a direct correlation to environmental sources.

5.2. Limitation and Future Work

Limitations and future work of this dissertation were alluded to in section 5.1. This includes: 1) sample size, allocation and timing; 2) sophistication of molecular tools; 3) no recount of

viability; and 4) limited information available in the region.

1. **Sample size, allocation and timing** is a limitation because it impeded on the ability to obtain vital information regarding *Cryptosporidium* population structure with geographical, temporal and statistical significance. The dataset as a whole consisted of biofilm samples collected from 5 sites, and 119 cattle feces collected from 10 farms; however, information for each sample location was restricted due to low occurrences of *Cryptosporidium* species and genotypes. Also, the samples were collected at different times, which contributed to insufficient epidemiological design. Although restrictions in funding made it unfeasible to increase sample size, it is also recognized that an alternative approach would have been to limit sample allocations to only a few water sources and farms within closer proximity. This would have potentially increased detection numbers within a smaller geographical location and timeframe, and thus help to draw stronger connections between *Cryptosporidium* transmission from cattle farms to nearby water sources. Future research should aim to examine these relationships at a more refine level, in order to gather geospatial information for source tracking *Cryptosporidium spp.* throughout the watershed.

2. **Sophistication of molecular tools** is a limitation because it restricted the ability to identify co-existing *Cryptosporidium spp*. within a single biofilm sample or co-infections within individual cattle specimens. It also restricted the sensitivity of molecular detection from environmental samples containing naturally low numbers of oocysts and inhibitors. As previously mentioned, superimposition was believed to have occurred during 18S rRNA and gp60 sequencing, where dominant species, in this case *C. parvum* and *C. hominis*, potentially overlaid the less dominant species (section 5.1.6.). Identification of

less dominant species requires sophisticated molecular tools such as multi-locus sequencing (the use of multiple gene targets) and PCR-RFLP to distinguish co-existing species or co-infections. In addition, advance molecular applications such as cloning or *in vitro* inoculation of positive samples into mice would have increased concentrations; thereby facilitate the identification of *Cryptosporidium* species and genotypes. In hindsight, improved identification of less dominant species using the same molecular tools employed in this dissertation, could have involved increasing the number of replicates per source. For example, successive sampling (e.g., sampling the same group of cattle multiple times over the duration of one month), would have accounted for intermittent infections.

3. **No account of viability** is a limitation because it did not provide information about whether the positive detections were viable; therefore, could cause infection. To a certain degree, knowledge of species and genotypes assemblages in surface water provides an understanding as to whether the population structure poses an immediate health risk. However, to be more precise, viability is an important factor to consider, especially when detecting *Cryptosporidium* from environmental sources because many oocysts could have been dead or damaged, thus do not pose significant threat to public health. Therefore, from a public health standpoint, viability is important to consider when making assessments about risk. Future research may investigate viability through various means including CC-PCR, bio-assays in mice, and immunofluorescence staining using vital dyes in conjunction with confocal microscopy to visualize occurrence of excystation.

4. **Limited information available in the region** presented major knowledge gaps regarding

host range and transmission dynamics. With no existing data on whether certain water sources accounted for outbreaks in the past, as well as records of livestock and wildlife causing zoonosis, it becomes difficult to assert whether cattle contributed as a point source of *Cryptosporidium spp.* loading into the Black River network. Even though the same *C. parvum* isolate detected in biofilm was found in cattle with >99% homology, other livestock animals within the watershed could have accounted for the transmission. Future work should therefore aim to establish databases of past anthropocentric occurrences of cryptosporidiosis, as well as species and genotypes detected from livestock and wildlife in Jamaica.

5.3. Summary of Research Contributions and Outlook

In situ biofilm sampling can produce biofilms at optimal thickness and roughness for oocysts entrapment in environmental waters. However, the randomized influence of sloughing and oocysts attachment to biofilms most likely was impacted by various factors including biofilm architecture, water chemistry and velocity, the diversity of organisms, and the presence of NOM. Thus, the biofilm sampling method warrants further investigation to obtain a better understanding of the complexity of biofilm development in natural waters and oocysts recovery efficiencies. This would make the technique more applicable for determining *Cryptosporidium* prevalence in a given region. Nonetheless, the technique was shown to provide informative data about the absence and presence of *Cryptosporidium.* Additionally, the technique provided a source of *Cryptosporidium* oocysts, which was effectively used for further molecular characterizations.

In terms of molecular analyses, initial research carried out in this dissertation used selected

primers previously published, as a basis for optimizing *Cryptosporidium* detection from environmental sources. Through spectrophotometric analysis and DNA gradient tests, a systematic process, which enhanced the sensitivity and specificity of conventional and nested-PCR was initiated. The procedure developed in this dissertation has shown to be effective for isolating enriched *Cryptosporidium* DNA from biofilm samples, thus is valuable for advanced genotyping assays that require pure and concentrated DNA. Moreover, verification of DNA purity was confirmed through successful 18S rRNA sequencing. Results have demonstrated that in conjunction with IMS, the genotyping assay generated the specificity and sensitivity needed for analyzing *Cryptosporidium* from biofilms. The use of this approach led to the 18S rRNA sequencing of a single *C. parvum* isolate in 4 PCR-positive biofilm samples. However, the downfall was the techniques inability to differentiate diverse *Cryptosporidium* species and genotypes, especially with further gp60 analyses. Therefore, with further refinement, the technique can potentially lead to the wide use of comparative genomics in epidemiological investigations of *Cryptosporidium* contamination in various water sources.

With regards to *Cryptosporidium* detection and gene sequencing from cattle feces, *C. hominis* and *C. parvum* were identified, which marks the first reported cases of *Cryptosporidium spp.* found in cattle feces from Jamaica. The identified species are the two most commonly found in humans; thus, this research provides evidence that there is potential zoonotic transmission occurring in the region. In addition, most *Cryptosporidium* detections were from dairy animals, which further supports the notion that there is a high prevalence of infections in dairy cattle compared to beef cattle. In the present study, restricted space of dairy herds was believed to have accounted for the higher prevalence of infections. Although, there are many other plausible factors to consider, thus further epidemiological research is necessary to draw this correlation.

Overall, our understanding of *Cryptosporidium* as an infectious protozoan parasite is influenced by previous researchers who have independently developed different methods for detecting and identifying *Cryptosporidium* species and genotypes, while drawing relationships between what was observed to 'real-life' occurrences. Although these novel methods have provided significant insight into *Cryptosporidium* biology and host-parasitic interactions, they have also led to a plethora of publications aiming to enhance the techniques, making it extremely difficult to establish a standardized method.

Many studies have stressed that standardization is imperative, to improve water-monitoring regimes from environmental sources (Cacci & Chalmers, 2016; Karanis et al., 20006; Díaz-Lee et al., 2015). However, it seems that no matter what innovative technique is employed for *Cryptosporidium* purification and detection, available technologies will continue to yield, to some degree, a level of inefficiency due to the abundance of inhibitors and naturally low number of oocysts found in environmental samples. Thus, the broader scope of this research calls for a shift in conventional monitoring frameworks for *Cryptosporidium* and alike species from environmental waters. Research should stray away from looking to adopt a single, standardized method; to incorporate a more integrated approach involving identification from multiple sources using several techniques. As long as there's an understanding of the sensitivity and specificity of each method, this collective approach could potentially be more applicable for obtaining a compressive representation of *Cryptosporidium* species and genotypes that exist in aquatic ecosystems.

As previously mention, the purpose of developing the *biofilm sampler* technique was to offer an alternative method to work in conjunction with multiple techniques applied to a group of sources that are known reservoirs or vectors of *Cryptosporidium spp*. Whether this group contains

biofilms, shellfishes, benthic macroinvertebrates, the idea is to improve recovery of oocysts from these natural sources because they are more reflective of the species and genotypes that persist at a sampling location compared to filtered grab-samples. Therefore, identification of *Cryptosporidium* species and genotype from a well-planned set of environmental sources, may aid in watershed monitoring, and thus develop interventions going forward.

Appendices

Appendix A: Suppliers and Catalogue Numbers of Reagents and Kits

Table A.I.: Reagents and Kits for IMS-IFA Staining

Reagents/Kits	Supplier (Cat. No.)
Dynabeads™ anti-*Cryptosporidium* Kit	Thermo Fisher Scientific, Waltham, MA (73011)
Crypt-a-Glo™ G/C Direct Comprehensive Kit	Waterborne, Inc., New Orleans, LA (A400FLK)
Hydrochloric acid	Thermo Fisher Scientific, Waltham, MA (SA54-1)
Sodium hydroxide solution	Thermo Fisher Scientific, Waltham, MA (SS266-1)
Methanol	Anachemia Canada, Lachine, QC (ANAC56902-360)
Nuclease-free Water	New England Biolabs Ltd., Ipswich, MA (12931S)

Table A.II.: Reagents and Kit for PBS-Ether Sedimentation and MAF Staining

Reagents/Kit	Supplier (Cat. No.)
Phosphate Buffer Saline (PBS) Tablets	VWR, Mississauga, ON (97062-730)
VWR Life Science Water, Sterile, Purified, Molecular Biology Grade	VWR, Mississauga, ON (L0201-1000)
Ethyl Ether	Thermo Fisher Scientific, Waltham, MA (E134-1)
Methanol	Anachemia Canada, Lachine, QC (ANAC56902-360)
Ward's® Chemistry Acid-Fast Stain Kit	VWR, Mississauga, ON (470038-940)
C. parvum slide	VWR, Mississauga, ON (470181-942)
C. parvum Non-viable Positive Control	Waterborne Inc., New Orleans, LA (PC101)

Table A.III.: Reagents for Biofilm Staining

Reagents	Supplier (Cat. No.)
SYTO™ 9 Green Fluorescent Nucleic Acid Stain	Thermo Fisher Scientific, Waltham, MA (S34854)
Streptavidin Texas Red™ Conjugates	Thermo Fisher Scientific, Waltham, MA (S872)

Table A.IV.: Reagents and Kit for ELISA

Kit	Supplier (Cat. No.)
CRYPTOSPORDIUM TEST	TechLab, Blacksburg, VA (PT5014)

Table A.V.: Reagents and Kits for DNA Extraction

Reagents/Kit	Supplier (Cat. No.)
Chelex ®100 Resin	Bio-Rad Laboratories, Inc., Hercules, Germany (1421253)
QIAamp® Fast DNA Stool Mini Kit	Qiagen, Hilden, Germany (51604)

Table A.VI.: Reagents and Kits for Conventional/Nested-PCR and Electrophoresis

Reagents/Kits	Supplier (Cat. No.)
Primers	ACGT Corporation, Toronto, ON
AccuStart™ II PCR ToughMix	Quantabio, Beverly, MA (95142-800)
FastStart™ Taq DNA Polymerase, dNTPack	Sigma-Aldrich, St. Louis, MO (4738357001)
GoTaq® DNA polymerase	Promega, Madison, WI (M3005)
DNA Gel Loading Dye (6X)	Thermo Fisher Scientific, Waltham, MA (R0611)
TAE Buffer	Thermo Fisher Scientific, Waltham, MA (15558042)
Ultra-Pure™ agarose	Invitrogent™, Carlsbad, CA (15510-027)
Ultra-Pure™ Ethidium Bromide	Thermo Fisher Scientific, Waltham, MA (15585011)
QIAxcel DNA Fast Analysis Kit	Qiagen, Hilden, Germany (929008)

Table A.VII.: Reagents and Kit for PCR Purification

Reagents/Kit	Supplier (Cat. No.)
QIAquick® PCR Purification Kit	Qiagen, Hilden, Germany (28104)

Table A.VIII.: Reagents and Kits for Gene Sequencing

Reagents/Kit	Supplier (Cat. No.)
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific, Waltham, MA (43374556)

Appendix B: Maps of the Black River Watershed

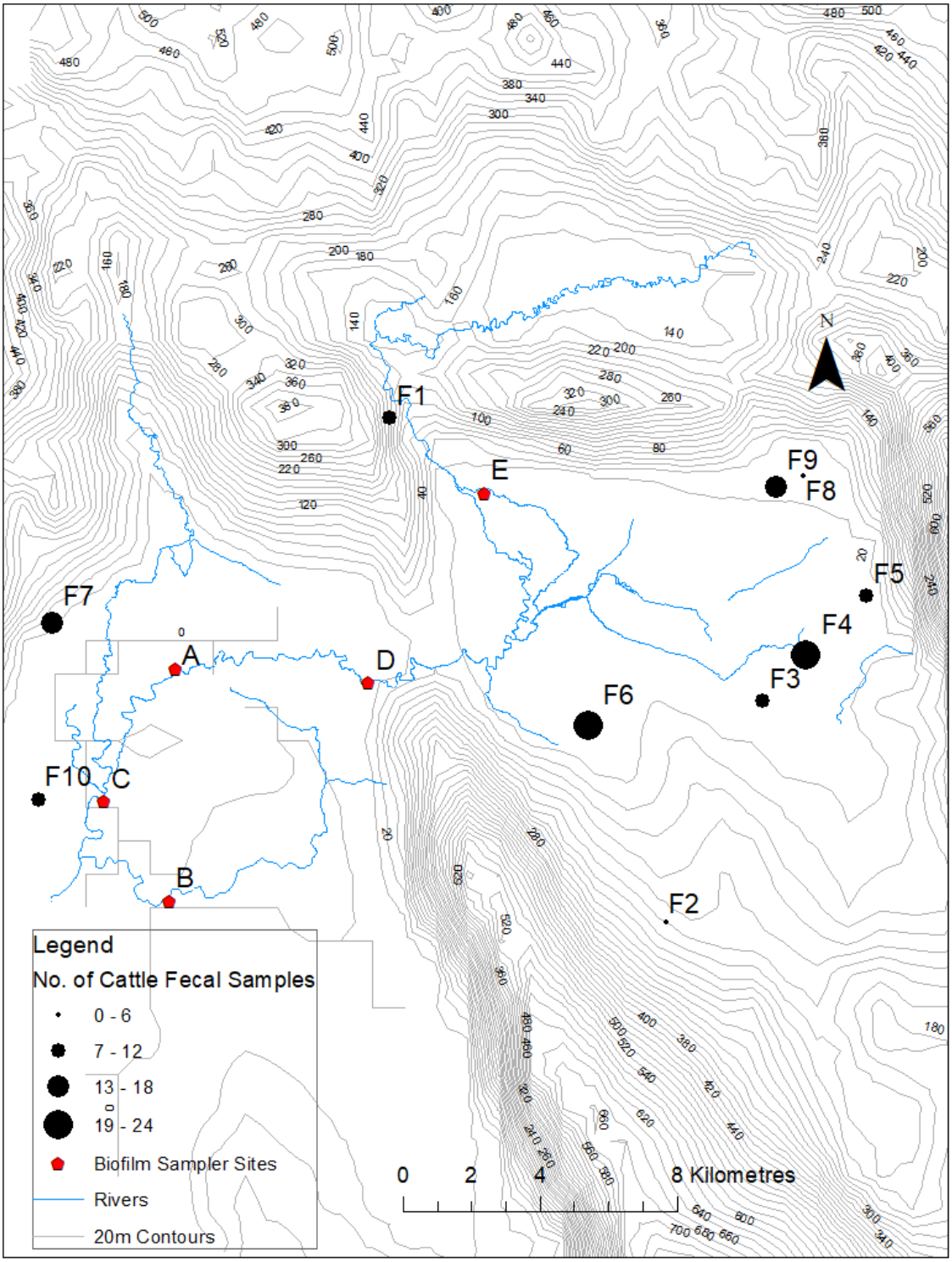


Figure B.I.: Topography map of the Black River watershed and sampling locations created in ARCMap10.

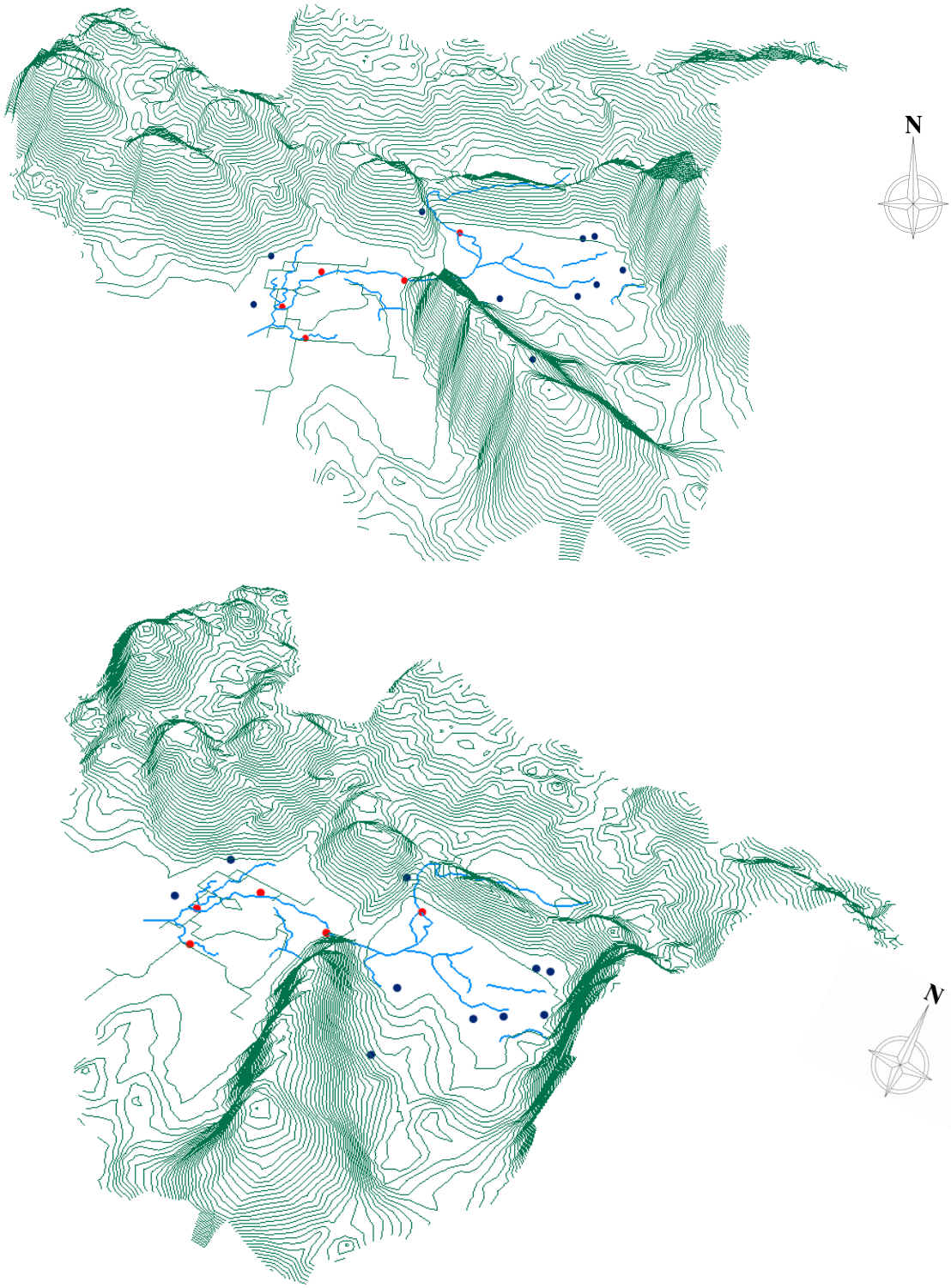


Figure B.II.: 3D Map of the Black River watershed from different point of views and sampling locations created in ARCScene10. *Biofilm sampler* sites (red) and cattle farms (blue).

Appendix C: Complete Data Sets

Table C.I.: Qualitative Records of Cattle Sample Collection

Farm I.D. and Coordinates	Cow I.D.	Collection Date	Approx. Age (yrs)	Sex	Breed (Production Type)	Diarrheal Symptoms
F1 (Lat: 18.14292; Long: -77.7578)	C1Cw	02/28/17	3	F	Indian (Beef)	Moderate
	C2Cw	02/28/17	4	F	Indian mixed with common (Beef)	None
	C3Cw	02/28/17	2	F	Indian mixed with common (Beef)	None
	C4Cw	02/28/17	0.5[a]	M	Brahman mixed with common (Beef)	None
	C5Cw	02/28/17	1	F	Indian (Beef)	None
	C6Cw	02/28/17	2.5	M	Brahman mixed with common (Beef)	Moderate
	77Cw	10/26/17	0.5[a]	F	Indian (Beef)	None
	91Cw	10/26/17	3	M	Brahman mixed with common (Beef)	Moderate
	94Cw	10/26/17	1.5	F	Indian mixed with common (Beef)	None
	111Cw	10/26/17	2.5	F	Indian (Beef)	Moderate
F2 (Lat: 18.017333; Long: -77.6853)	C7Cw	02/28/17	1	M	Red Poll mixed with common (Beef)	None
	107Cw	10/26/17	4	F	Indian (Beef)	None
	33BeCw	03/13/17	0.1[a]	M	Red Poll mixed with common (Beef)	None
	103Cw	10/26/17	1	F	Brahman (Beef)	None
	112Cw	10/26/17	0.5[a]	M	Red Poll mixed with common (Beef)	None
F3 (Lat: 18.072382; Long: -77.659925)	C19Cw	02/28/17	4	F	Jamaica Hope (Dairy)	None
	C20Cw	02/28/17	6	F	Jamaica Hope (Dairy)	None
	100Cw	10/26/17	8	F	Holstein (Dairy)	None
	102Cw	10/26/17	11	F	Holstein (Dairy)	Moderate
	105Cw	10/26/17	1	F	Holstein (Dairy)	Moderate
	106Cw	10/26/17	2	M	Red Poll (Beef)	Moderate
	114Cw	10/26/17	1	M	Red Poll (Dairy)	Severe
F4 (Lat: 18.083909; Long: -77.648551)	C8Cw	02/28/17	0.5[a]	M	Red Poll (Beef)	Severe
	C9Cw	02/28/17	15	F	Holstein (Dairy)	Moderate
	C10Cw	02/28/17	8	F	Holstein mixed with Jamaica Hope (Dairy)	None
	C11Cw	02/28/17	8	F	Jamaica Hope (Dairy)	None
	C12Cw	02/28/17	3	F	Jamaica Hope (Dairy)	None
	C13Cw	02/28/17	4	F	Jamaica Hope (Dairy)	None
	C14Cw	02/28/17	3	F	Jamaica Hope (Dairy)	None
	C15Cw	02/28/17	3	F	Jamaica Hope (Dairy)	None
	46BeCw	03/13/17	3	M	Red Poll (Beef)	None
	47BeCw	03/13/17	1.5	M	Indian (Beef)	None
	48Cw	03/13/17	0.25[a]	M	Black Poll (Beef)	None
	49BeCw	03/13/17	4	F	Indian mixed with common (Beef)	Moderate

	50BeCw	03/13/17	5	F	Red Poll (Beef)	None
	51BeCw	03/13/17	2.5	M	Black Poll (Beef)	None
	54BeCw	03/13/17	0.5[a]	M	Black Poll (Beef)	Severe
	68Cw	03/13/17	5	F	Jamaica Hope (Dairy)	None
	40DaCw	03/13/17	4	F	Jamaica Hope (Dairy)	Moderate
	72Cw	03/13/17	3.5	F	Jamaica Hope (Dairy)	None
	74Cw	03/13/17	3.5	M	Indian mixed with common (Dairy)	None
	88Cw	10/26/17	4	F	Jamaica Hope (Dairy)	None
F5 (Lat: 18.098494; Long: -77.632647)	C16Cw	02/28/17	9	F	Holstein (Dairy)	Moderate
	C17Cw	02/28/17	11	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
	C18Cw	02/28/17	9	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
	C21Cw	02/28/17	7	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
	36DaCw	03/13/17	9	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
	79Cw	10/26/17	8	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
	87Cw	10/26/17	7	F	Holstein mixed with Jamaica Hope (Dairy)	Moderate
F6 (Lat: 18.066185; Long: -77.705726)	C22Cw	02/28/17	0.5[a]	M	Indian mixed with common (Beef)	None
	C23Cw	02/28/17	1	M	Indian mixed with common (Beef)	None
	C24Cw	02/28/17	1.5	F	Red Poll (Beef)	None
	C25Cw	02/28/17	0.5[a]	M	Indian mixed with common (Beef)	None
	C26Cw	02/28/17	2	M	Red Poll (Beef)	None
	39BeCw	03/13/17	1.5	F	Red Poll (Beef)	None
	80Cw	10/26/17	0.7	M	Red Poll mixed with common (Beef)	None
	83Cw	10/26/17	0.75	M	Indian mixed with common (Beef)	None
	85Cw	10/26/17	0.9	M	Indian mixed with common (Beef)	None
	86Cw	10/26/17	0.5[a]	M	Indian mixed with common (Beef)	None
	99Cw	10/26/17	1.5	F	Red Poll (Beef)	None
	101Cw	10/26/17	0.3[a]	F	Indian mixed with common (Beef)	None
	108Cw	10/26/17	2	M	Red Poll (Beef)	None
	109Cw	10/26/17	1	M	Indian mixed with common (Beef)	None
	110Cw	10/26/17	1.5	M	Indian (Beef)	Moderate
	115Cw	10/26/17	3	F	Red Poll (Beef)	None
	116Cw	10/26/17	5	F	Indian mixed with common (Beef)	None
	117Cw	10/26/17	3.5	F	Red Poll (Beef)	Moderate
	118Cw	10/26/17	0.1[a]	M	Indian mixed with common (Beef)	Severe
	119Cw	10/26/17	0.5[a]	M	Red Poll (Beef)	None
F7 (Lat:18.091868; Long: -77.84639)	C27Cw	02/28/17	2	M	Jamaica Hope (Dairy)	Severe
	C28Cw	02/28/17	0.2[a]	F	Jamaica Hope (Dairy)	None
	C29Cw	02/28/17	0.1[a]	F	Jamaica Hope (Dairy)	Diarrhea
	92Cw	10/26/17	7	F	Jamaica Hope (Dairy)	Diarrhea

	104Cw	10/26/17	0.1[a]	F	Jamaica Hope (Dairy)	Diarrhea
	52DaCw	03/13/17	1.5	F	Jamaica Hope (Dairy)	None
	53DaCw	03/13/17	0.2[a]	F	Jamaica Hope (Dairy)	Diarrhea
	55DaCw	03/13/17	5	F	Jamaica Hope (Dairy)	Diarrhea
	56DaCw	03/13/17	1	F	Jamaica Hope (Dairy)	Diarrhea
	57DaCw	03/13/17	3.5	F	Jamaica Hope (Dairy)	None
	58DaCw	03/13/17	0.5[a]	F	Jamaica Hope (Dairy)	Diarrhea
	59DaCw	03/13/17	5	F	Jamaica Hope (Dairy)	None
	60DaCw	03/13/17	3	F	Jamaica Hope (Dairy)	None
	61DaCw	03/13/17	2	F	Jamaica Hope (Dairy)	None
	95Cw	10/26/17	2	M	Jamaica Hope (Dairy)	None
	73Cw	03/13/17	0.2[a]	F	Jamaica Hope (Dairy)	None
	89Cw	10/26/17	0.7	F	Jamaica Hope (Dairy)	None
	90Cw	10/26/17	3	F	Jamaica Hope (Dairy)	None
F8 (Lat: 18.128619; Long: -77.649231)	C30Cw	02/28/17	0.5[a]	F	Indian (Beef)	None
	C31Cw	02/28/17	1.5	F	Black Poll (Beef)	None
	113Cw	02/28/17	3	F	Indian (Beef)	Diarrhea
	70Cw	03/13/17	4	F	Indian mixed with common (Beef)	None
	71Cw	03/13/17	2	F	Black Poll (Beef)	None
	82Cw	10/26/17	0.5[a]	M	Brahman mixed with common (Beef)	None
F9 (Lat: 18.125799; Long: -77.656546)	C32Cw	03/13/17	1	F	Red Poll mixed with common (Beef)	Diarrhea
	35DaCw	03/13/17	0.5[a]	M	Jamaica Hope (Dairy)	None
	34DaCw	03/13/17	2	F	Holstein (Dairy)	Diarrhea
	69Cw	03/13/17	1.5	F	Red Poll (Beef)	None
	84Cw	03/13/17	5	F	Red Poll (Beef)	None
	38BeCw	03/13/17	4	F	Indian mixed with common (Beef)	None
	97Cw	10/26/17	2.5	F	Holstein (Dairy)	Diarrhea
	41DaCw	03/13/17	1.5	F	Jamaica Hope (Dairy)	None
	42DaCw	03/13/17	6	M	Holstein (Dairy)	None
	43DaCw	03/13/17	2.5	F	Holstein (Dairy)	Diarrhea
	44DaCw	03/13/17	2	F	Jamaica Hope (Dairy)	None
	45DaCw	03/13/17	1.5	F	Jamaica Hope (Dairy)	None
	37BeCw	03/13/17	4	M	Black Poll (Beef)	None
	81Cw	10/26/17	1	F	Red Poll mixed with common (Beef)	Diarrhea
	76Cw	03/13/17	0.25[a]	F	Jamaica Hope (Dairy)	None
	78Cw	03/13/17	1.5	F	Jamaica Hope (Dairy)	None
	62DaCw	03/13/17	2	F	Holstein (Dairy)	None
	63DaCw	03/13/17	2	F	Jamaica Hope (Dairy)	None
	64BeCw	03/13/17	3	M	Brahman (Beef)	Diarrhea

F10 (Lat: 18.047851; Long: -77.849876)	65DaCw	03/13/17	2	F	Jamaica Hope (Dairy)	None
	66DaCw	03/13/17	3	F	Holstein (Dairy)	None
	67DaCw	03/13/17	5	F	Holstein (Dairy)	None
	75Cw	03/13/17	3	M	Brahman (Beef)	Diarrhea
	93Cw	10/26/17	2	F	Jamaica Hope (Dairy)	None
	96Cw	10/26/17	2	F	Black Poll (Beef)	None
	98Cw	10/26/17	6	M	Holstein (Dairy)	None

[a] Pre-weaned calf; 0 to 0.5 yr (*n*=23)
0.6 to 1 yr (*n*=26)
2 to 3 yr (*n*=37)
4 to 5 yr (*n*=17)
≥ 6 yr (*n*=16)

Table C.II.: MAF/ELISA/PCR Detections of *Cryptosporidium* in Cattle Feces According to Age, Production Type and Diarrheal Symptom

Farm	Cow ID	MAF	ELISA	PCR	Age	Production Type	Diarrheal Symptoms
F1	C2Cw	+			4	Beef	None
F1	C5Cw	+	+		1	Beef	None
F1	C6Cw	+	+		2.5	Beef	Moderate
F3	105Cw	+	+	+	1	Dairy	Moderate
F4	C8Cw	+	+	+	0.5[a]	Beef	Severe
F4	C11Cw	+	+		8	Dairy	None
F4	C13Cw		+	+	4	Dairy	None
F4	46BeCw	+		+	3	Beef	None
F4	51BeCw	+			2.5	Beef	None
F4	68Cw		+		5	Dairy	None
F4	72Cw		+		3.5	Dairy	None
F4	74Cw	+	+		3.5	Dairy	None
F4	40DaCw		+	+	4	Dairy	Moderate
F4	88Cw	+			4	Dairy	None
F5	C18Cw	+			9	Dairy	Moderate
F5	79Cw	+			8	Dairy	Moderate
F6	C24Cw	+			1.5	Beef	None
F6	101Cw		+		0.3[a]	Beef	None
F6	116Cw	+			5	Beef	None
F7	C28Cw	+	+		0.2[a]	Dairy	None
F7	53DaCw	+	+	+	0.2[a]	Dairy	Moderate
F7	55DaCw	+	+		5	Dairy	Moderate
F7	58DaCw	+	+		0.5[a]	Dairy	Moderate
F7	61DaCw		+		2	Dairy	None
F7	73Cw	+			0.2[a]	Dairy	None

F7	89Cw	+	+		0.7	Dairy	None
F7	56Da	+	+	+	1	Dairy	Moderate
F7	60Da	+			3	Dairy	None
F7	95Cw	+			2	Dairy	None
F8	C31Cw	+	+		1.5	Beef	None
F8	70Cw	+			4	Beef	None
F9	34DaCw	+	+	+	2	Dairy	Moderate
F9	38BeCw		+		4	Beef	None
F9	42DaCw	+			6	Dairy	None
F9	78Cw		+	+	1.5	Dairy	None
F10	64BeCw	+		+	3	Beef	Moderate
F10	65DaCw	+			2	Dairy	None
F10	75Cw		+		3	Beef	Moderate
F10	98Cw		+		6	Dairy	None
Total Positive	**-**	**29**	**24**	**10**	**-**	**-**	**-**

[a] Pre-weaned calf

Table C.III.: Cattle Population Density per Hectare of Land in St. Elizabeth as of October 2017

District	**Beef**		**Dairy**	
	Hectare of Land	**Count**	**Hectare of Land**	**Count**
Balaclava	519.7	1167	85.4	276
Black River	2877.56	3468	420.16	987
Braes River	1214.6	1890	114.19	550
Ginger Hill	1319.8	1067	8.69	22
Ipswich	358.96	277	0	0
Junction	329.96	466	18	38
Lacovia	1065.96	1511	6.74	50
Malvern	725.08	921	73.23	202
Mountainside	2064.77	4188	99.44	395
New Market	634.46	891	28.93	21
Pedro Plains	660.35	801	40.33	152
Santa Cruz	1339.41	2580	30.84	70
Southfield	110.67	241	3.84	5
Total	**13221.28**	**19468**	**929.73**	**2768**

Note: Data set provide by Jamaica RADA (2017)

Table C.IV.: Farm Management Data

Farm I.D.	Production	Drinking Water Source(s)	Land Rearing/ Flooring System	Hygiene Maintenance of Pen or Land	Cow-calf Separation
F1	– beef	– pipe[a], surface water, and rain	– grass rearing – no fencing	– feces cleared from land 1-2 times per week	– no; cows are tied to tree/peg with rope and pre-weaned calves roam freely
F2	– beef	– pipe[a] and rain	– grass rearing – fencing	– none	– no; cows and calves are freely roaming
F3	– beef and dairy (raised separate)	– pipe[a] and rain	– concrete and grass rearing – fencing	– feces cleared from land daily – concrete pen is washed daily with water and occasionally with disinfectant	– yes; cows freely roam on grass and pre-weaned calves are raised on concreate
F4	– beef and dairy (raised separate)	– pipe[a], surface water (beef), and rain	– concrete/dirt and grass rearing – partial fencing	– feces cleared from land 2-3 times per week – concrete pen is washed daily with water and weekly with disinfectant	– yes; cows freely roam on grass and pre-weaned calves are raised on concreate/dirt
F5	– dairy	– pipe[a] and rain	– grass and gravel rearing – fencing	– feces cleared from pen multiple times per day	– yes; freely roaming, but adult cows are separated from calves
F6	– beef	– pipe[a] and rain	– grass rearing – partial fencing	– none	– no; cows are tied to tree/peg with rope and pre-weaned calves roam freely
F7	– dairy	– pipe[a] and rain	– grass rearing – fencing	– feces are cleared multiple times per day	– yes; freely roaming, but pre-weaned, young and adult cows are separated

F8	– beef	– pipe[a] and rain	– concrete and grass rearing – fencing	– none	– no
F9	– beef and dairy (raised separate)	– pipe[a] and rain	– concrete and grass rearing – fencing	– feces are not cleared from grassland – concrete pen is washed daily with disinfect	– no
F10	– beef and dairy (raised together)	– surface water[a], and rain	– grass and gravel rearing – fencing	– none	– no; cows and calves roam freely in enclosed area

[a] primary drinking water source

Table C.V.: COMSTAT2 Analyses of Biofilm Segments

Biofilm I.D.	Replicate Biofilm Segments	Average Biofilm Thickness	Maximum Biofilm Thickness	Roughness Coefficient
Bply1	R1	34.87	62	1.87
	R2	20.43	86	1.96
	R3	6.21	52	1.98
	R4	68.84	126	1.52
	R5	33.61	94	1.85
	R6	49.47	112	1.7
	R7	69	111	1.35
	R8	104.2	132	1.88
Bply2	R1	34.98	108	1.82
	R2	36.1	87	1.9
	R3	10.72	35	1.26
	R4	83.34	116	1.86
	R5	66.4	104	2.01
	R6	82.14	113	1.77
	R7	25.23	98	1.36
	R8	83.3	104	1.89
Bply3	R1	101.35	128	1.66
	R2	68.46	111	1.38
	R3	57.36	97	1.89
	R4	87.2	131	1.78
	R5	98.21	109	2.13
	R6	71.58	92	2.19

	R7	109.36	136	1.79
	R8	66.71	117	1.51
Bply4	R1	35.4	94	1.64
	R2	101.6	113	1.39
	R3	52.69	123	1.44
	R4	82.5	105	1.72
	R5	23.6	88	1.8
	R6	10.4	62	1.92
	R7	80.3	104	1.83
	R8	97.23	114	1.36
Mean	**-**	**61.02**	**102**	**1.73**
S.D.	**-**	**30.37**	**22.98**	**0.24**

BglsREF	R1	23.36	32.64	0.21
	R2	4.32	17.98	0.64
	R3	17.06	36.05	0.36
	R4	28.14	30.15	0.31
	R5	26.35	43.21	0.48
	R6	11.34	39.35	0.13
	R7	19.43	26.25	0.42
	R8	7.68	16.28	0.44
Mean	**-**	**17.21**	**30.24**	**0.37**
S.D.	**-**	**8.19**	**9.03**	**0.15**

Table C.VI.: Recovery of *C. Parvum* Oocysts from Biofilm Suspensions by IMS

Biofilm I.D.	Replicate Microscope Slide	No. of oocysts Counted in Columns	Calculated Recovery (%)	Mean Turbidity (NTU)[a]
Bply1	R1	378	63.58	544.1 ± 24.72
	R2	459	77.21	
	R3	403	67.79	
	R4	394	66.27	
Bply2	R1	214	35.1	554.1 ± 26.81
	R2	227	38.18	
	R3	183	30.78	
	R4	211	35.5	
Bply3	R1	223	37.51	549.6 ± 23.64
	R2	408	68.63	
	R3	218	36.67	
	R4	195	32.8	

	R1	189	31.79	544.3 ± 27.14
	R2	136	22.88	
	R3	147	24.73	
Bply4	R4	96	16.15	
Mean	**-**	**246.87**	**42.9**	**548.1**
S.D.	**-**	**109.66**	**18.45**	**25.95**

[a] Mean ± S.D. (*n*=10)

Table C.VII.: Spectrophotometric (NanoDrop ND1000) Readings

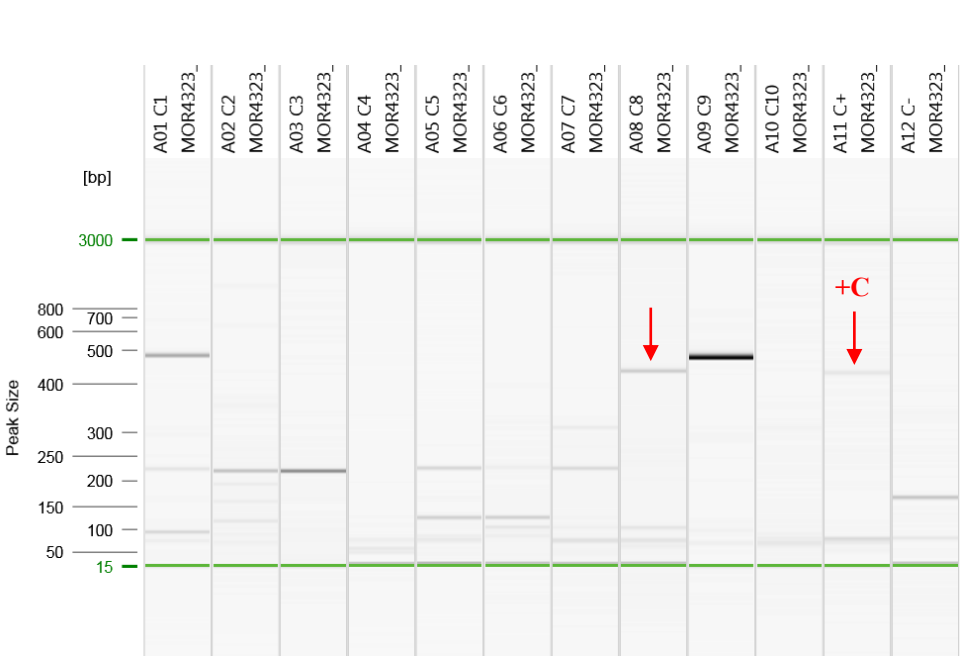
Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
C8Cw	Default	8/18/2017	12:14 PM	10.57	0.211	0.08	2.65	0.07	50	230	3.224	-0.144
C17Cw	Default	8/18/2017	12:15 PM	0.63	0.013	0.02	0.63	0.69	50	230	0.018	-0.002
D3Bio	Default	8/18/2017	12:26 PM	267.13	5.343	2.809	1.9	2.24	50	230	2.385	0.04
D2Bio	Default	8/18/2017	12:27 PM	319.11	6.382	3.371	1.89	2.27	50	230	2.809	0.048
CPB-DIAG F (100)	Default	8/18/2017	12:22 PM	642.31	19.464	12.71	1.53	1.41	33	230	13.792	39.045
CPB-DIAG R (100)	Default	8/18/2017	12:22 PM	925.65	28.05	13.61	2.06	2.71	33	230	12.58	0.064
dil CPB-DIAG F (5)	Default	8/18/2017	12:22 PM	35.67	1.081	0.712	1.52	2.63	33	230	0.468	0.026
dil CPB-DIAG R (5)	Default	8/18/2017	12:22 PM	42.12	1.283	0.831	1.54	2.14	33	230	0.435	0.037
DNA ctrl	Default	8/18/2017	12:18 PM	90.32	1.806	0.943	1.92	1.77	50	230	1.018	-0.029
NFW	Default	8/18/2017	12:28 PM	1.54	0.031	-0.02	-1.67	2.67	50	230	0.012	-0.001

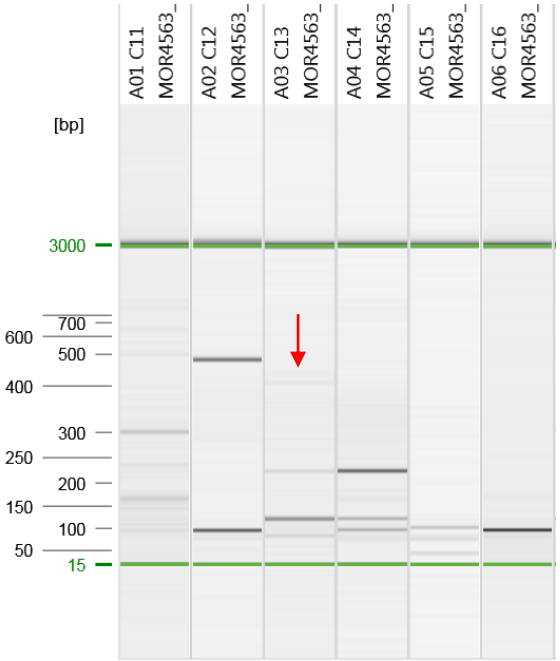
Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
KLJ F (100)	Default	10/05/2017	11:53 PM	718.15	21.813	14.212	1.53	2.31	33	230	12.84	0.049
KLJ F (100)	Default	10/05/2017	11:54 PM	639.58	19.401	12.673	1.53	2.22	33	230	13.25	0.075
dil KLJ F (5)	Default	10/05/2017	11:54 PM	37.89	1.147	0.76	1.51	2.19	33	230	0.502	0.033
dil KLJ R (5)	Default	10/05/2017	11:55 PM	36.54	1.108	0.93	1.19	2.17	33	230	0.567	0.011
DNA ctrl	Default	10/05/2017	11:56 PM	103.68	1.806	0.943	1.92	1.77	50	230	1.013	-0.029
NFW	Default	10/05/2017	11:56 PM	-0.82	-0.016	0.001	-10.1	0.67	50	230	0.031	-0.001

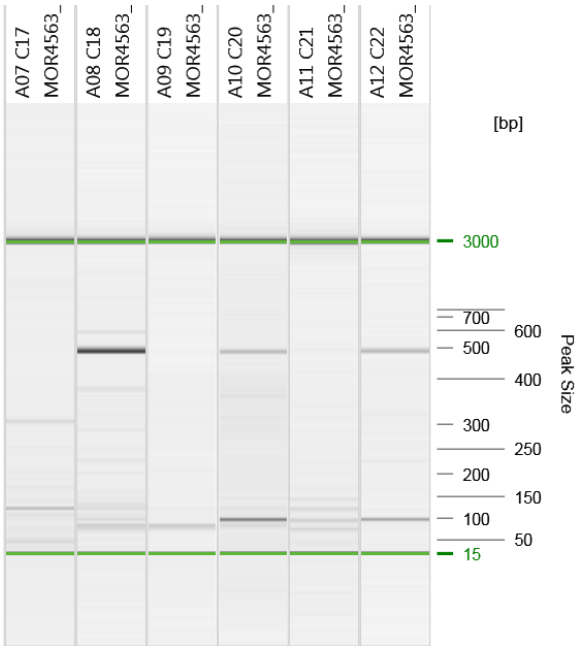
Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
XIAO 1F (100)	Default	7/12/2018	4:29 PM	552.59	16.745	9.084	1.84	2.39	33	230	7.001	0.097
XIAO 1R (100)	Default	7/12/2018	4:30 PM	596.4	18.073	10.613	1.7	2.1	33	230	8.614	0.062
XIAO 2F (100)	Default	7/12/2018	4:30 PM	736.67	22.323	10.835	2.06	2.93	33	230	7.627	0.152

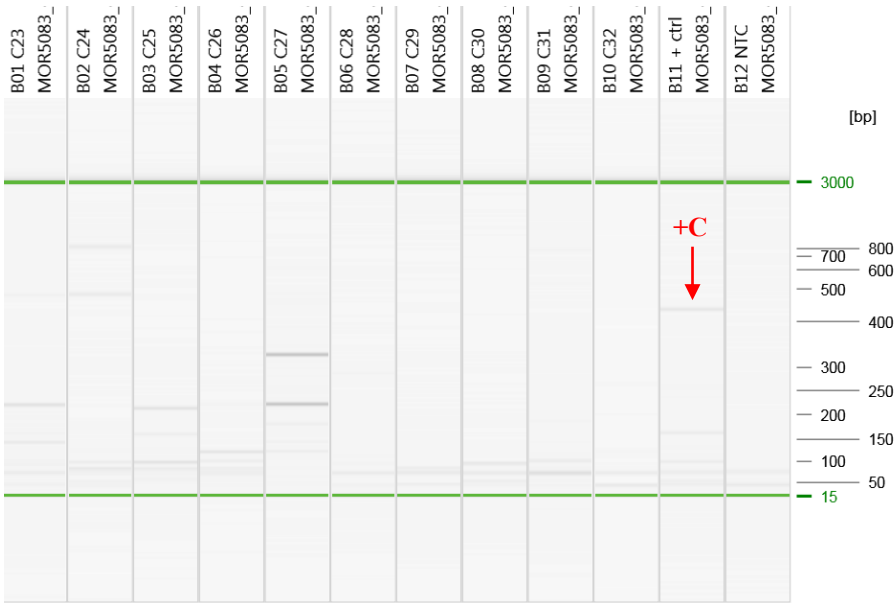
XIAO 2R (100)	Default	7/12/2018	4:31 PM	630.25	19.098	8.76	2.18	2.3	33	230	8.286	0.025
dil XIAO 1F (5)	Default	7/12/2018	4:32 PM	28.74	0.871	0.538	1.62	2.5	33	230	0.348	0.018
dil XIAO 1R (5)	Default	7/12/2018	4:32 PM	30.05	0.911	0.61	1.49	2.22	33	230	0.409	0.024
dil XIAO 2F (5)	Default	7/12/2018	4:33 PM	40.76	1.235	0.636	1.94	2.88	33	230	0.429	0.022
dil XIAO 2R (5)	Default	7/12/2018	4:33 PM	34.18	1.036	0.543	1.91	2.32	33	230	0.446	0.059
NFW	Default	7/12/2018	4:33 PM	0.87	0.026	0.062	0.42	0.83	33	230	0.031	0.02
LMP1 -1409	Default	7/12/2018	4:35 PM	11.03	0.221	0.123	1.79	2.05	50	230	0.108	-0.03
NFW	Default	7/12/2018	4:35 PM	-0.56	-0.011	0.001	-9.8	0.57	50	230	-0.02	0.006

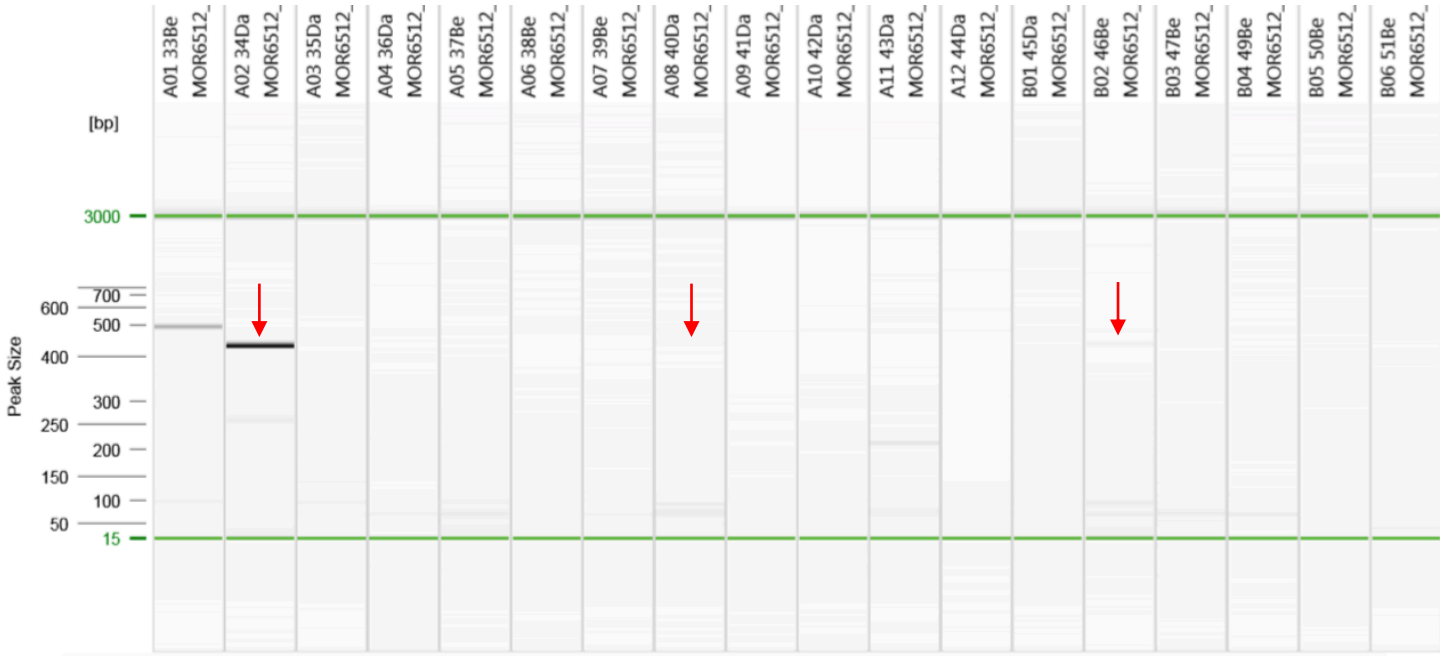
Appendix D: Electrophoresis Images of *Cryptosporidium* Detection from Cattle Feces and Positive Controls

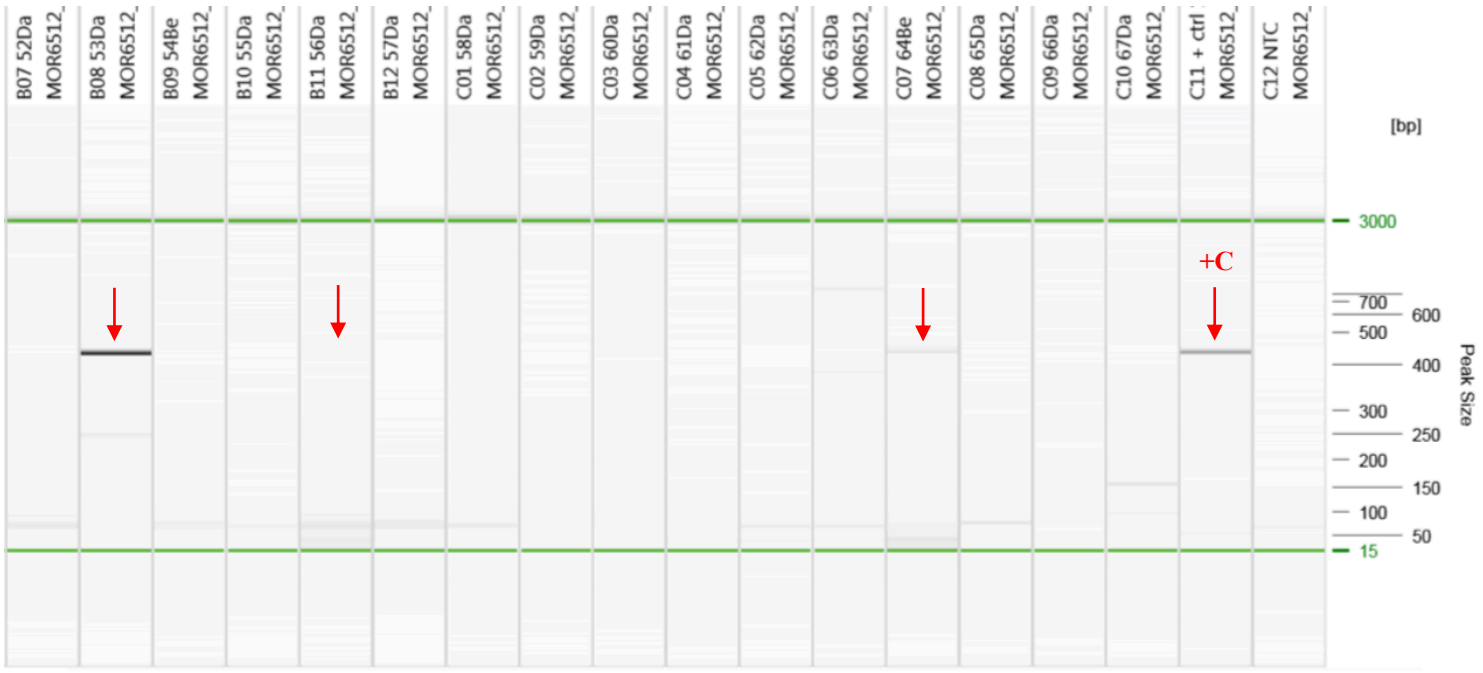


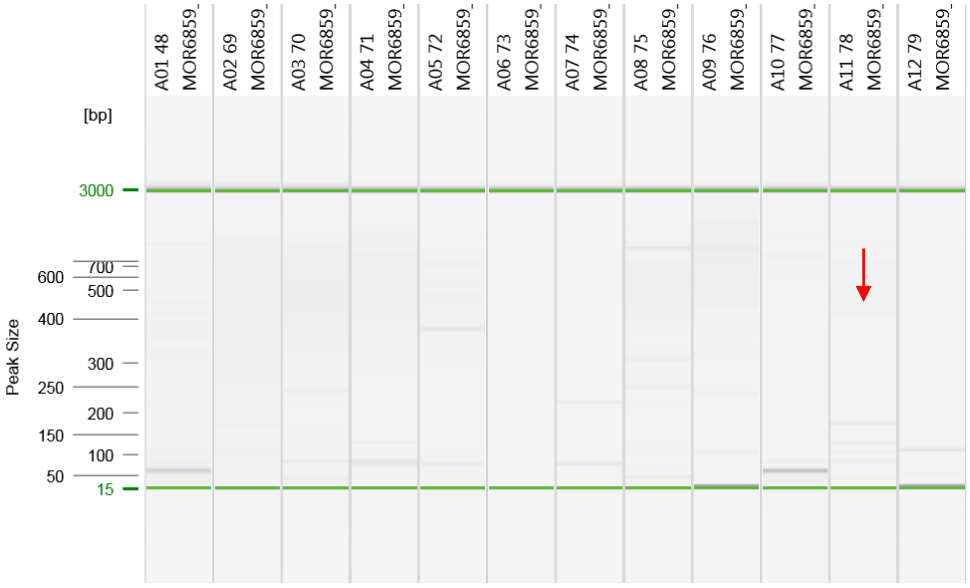


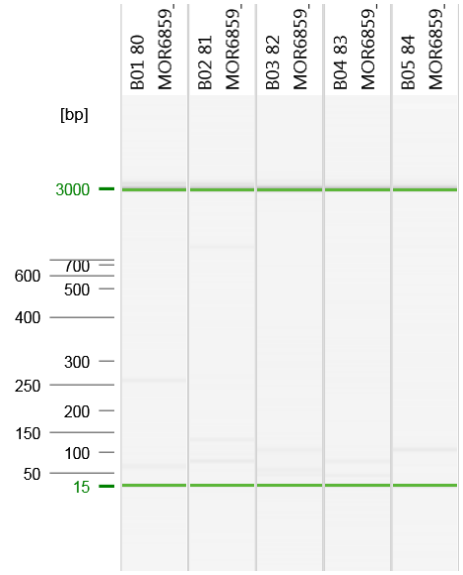


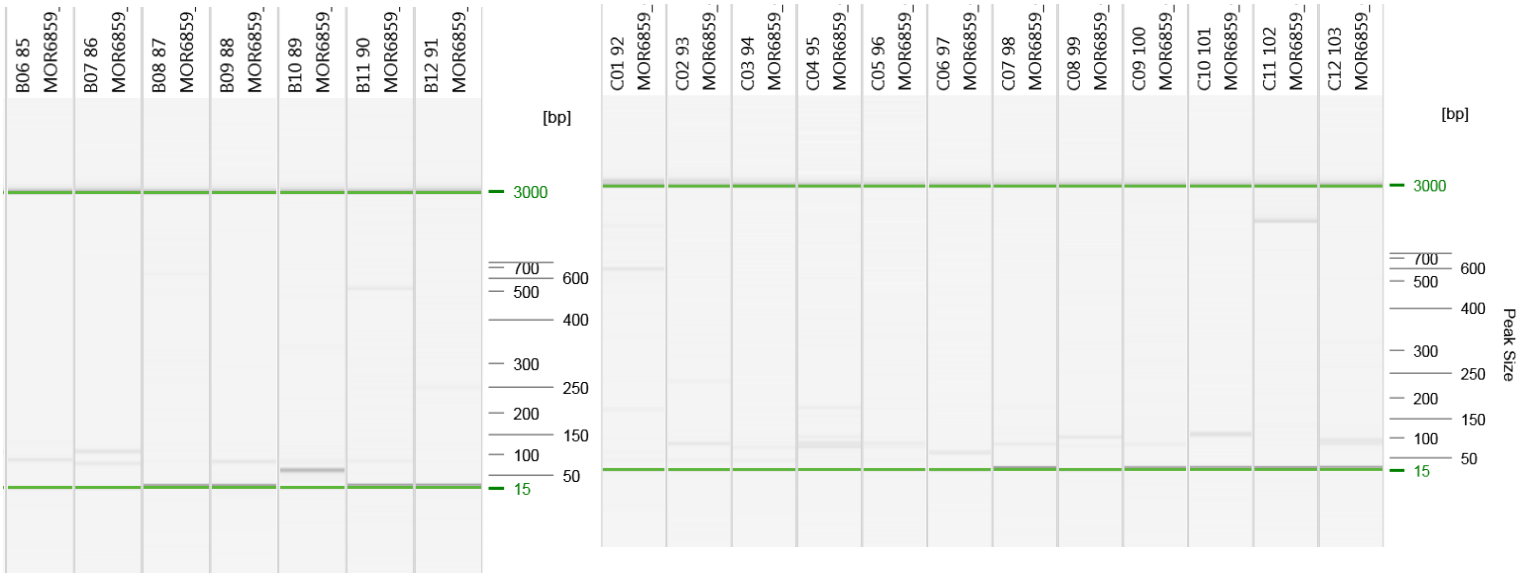



A01 33Be MOR6512_
A02 34Da MOR6512_
A03 35Da MOR6512_
A04 36Da MOR6512_
A05 37Be MOR6512_
A06 38Be MOR6512_
A07 39Be MOR6512_
A08 40Da MOR6512_
A09 41Da MOR6512_
A10 42Da MOR6512_
A11 43Da MOR6512_
A12 44Da MOR6512_
B01 45Da MOR6512_
B02 46Be MOR6512_
B03 47Be MOR6512_
B04 49Be MOR6512_
B05 50Be MOR6512_
B06 51Be MOR6512_
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15
Peak Size


B07 52Da MOR6512_
B08 53Da MOR6512_
B09 54Be MOR6512_
B10 55Da MOR6512_
B11 56Da MOR6512_
B12 57Da MOR6512_
C01 58Da MOR6512_
C02 59Da MOR6512_
C03 60Da MOR6512_
C04 61Da MOR6512_
C05 62Da MOR6512_
C06 63Da MOR6512_
C07 64Be MOR6512_
C08 65Da MOR6512_
C09 66Da MOR6512_
C10 67Da MOR6512_
C11 + ctrl MOR6512_
C12 NTC MOR6512_
+C
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15
Peak Size


A01 48 MOR6859_
A02 69 MOR6859_
A03 70 MOR6859_
A04 71 MOR6859_
A05 72 MOR6859_
A06 73 MOR6859_
A07 74 MOR6859_
A08 75 MOR6859_
A09 76 MOR6859_
A10 77 MOR6859_
A11 78 MOR6859_
A12 79 MOR6859_
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15
Peak Size


B01 80 MOR6859_
B02 81 MOR6859_
B03 82 MOR6859_
B04 83 MOR6859_
B05 84 MOR6859_
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15


B06 85 MOR6859_
B07 86 MOR6859_
B08 87 MOR6859_
B09 88 MOR6859_
B10 89 MOR6859_
B11 90 MOR6859_
B12 91 MOR6859_
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15
C01 92 MOR6859_
C02 93 MOR6859_
C03 94 MOR6859_
C04 95 MOR6859_
C05 96 MOR6859_
C06 97 MOR6859_
C07 98 MOR6859_
C08 99 MOR6859_
C09 100 MOR6859_
C10 101 MOR6859_
C11 102 MOR6859_
C12 103 MOR6859_
[bp]
3000
700
600
500
400
300
250
200
150
100
50
15
Peak Size

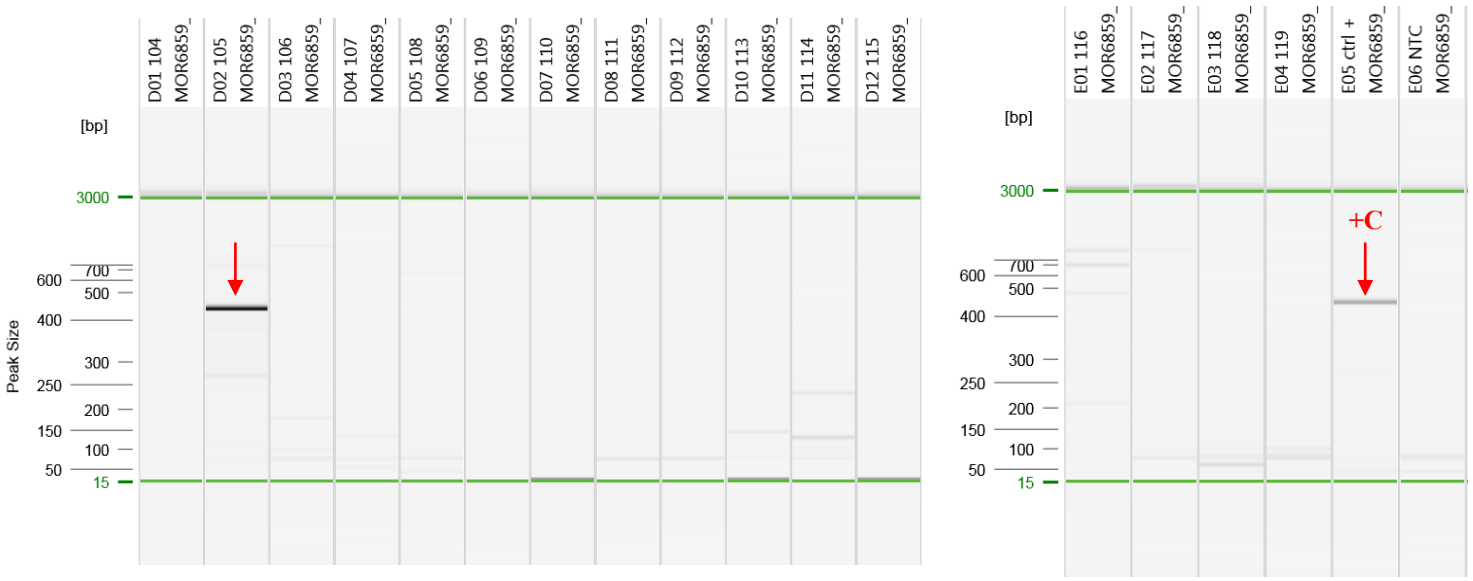
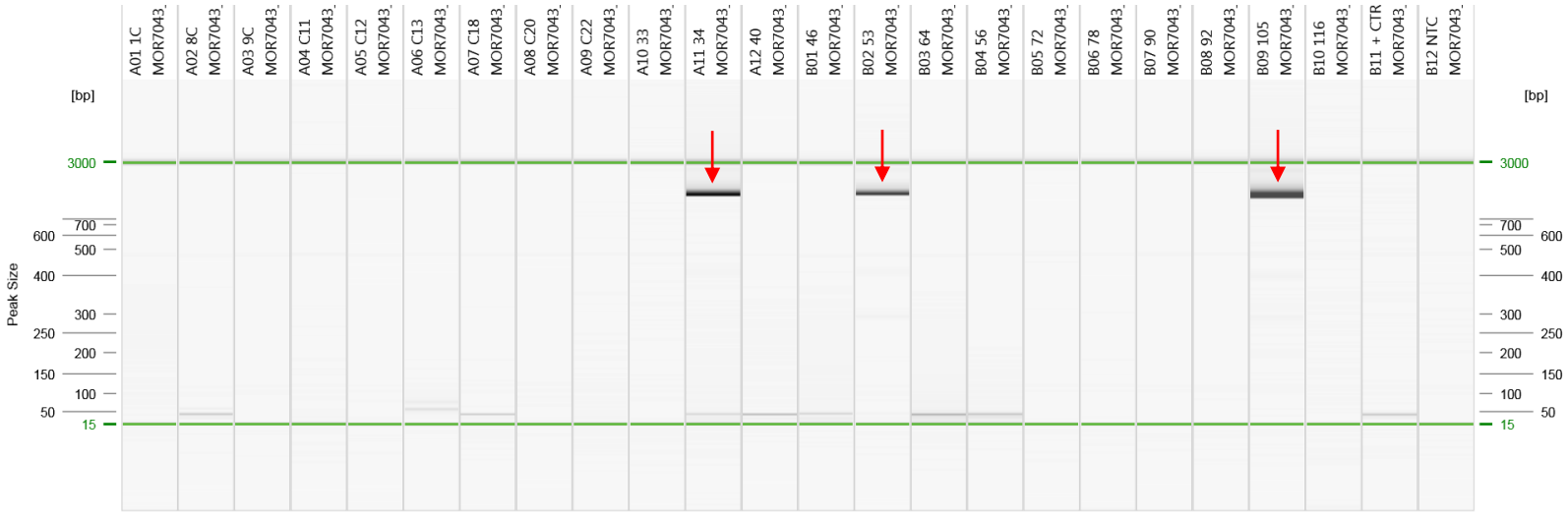


Figure D.I.: Electrophoresis images of *Cryptosporidium* detection derived from conventional PCR products targeting the 435 bp of the 18S rRNA.

First Round



Second Round

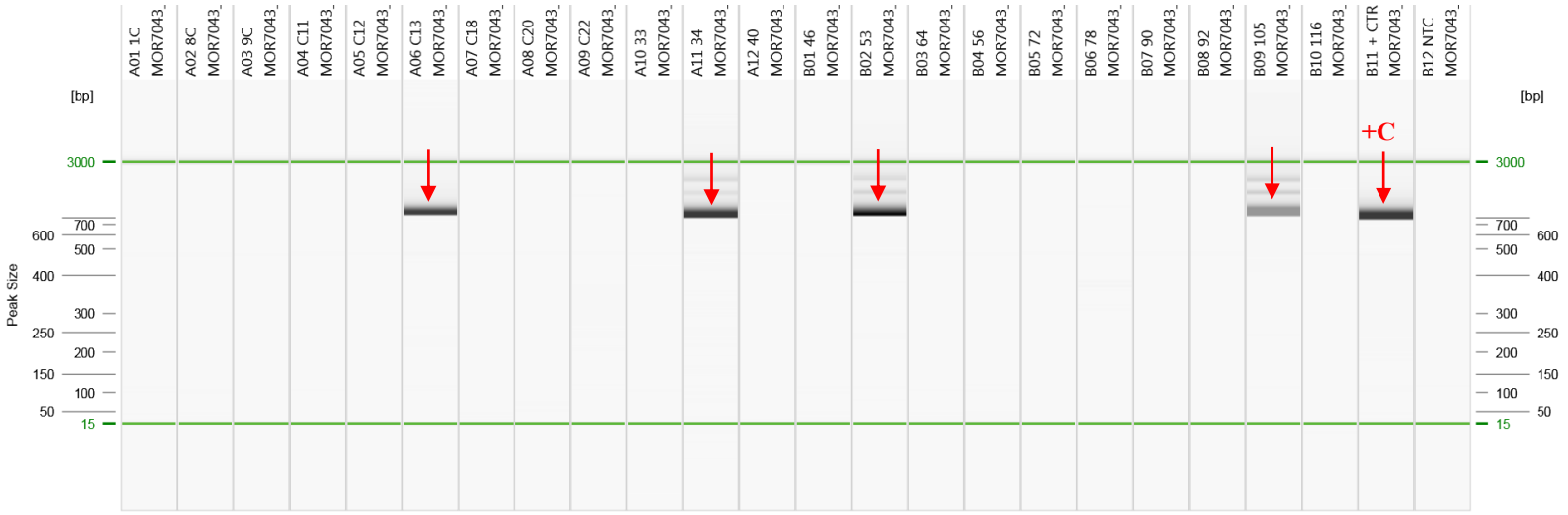


Figure D.II.: Electrophoresis images of *Cryptosporidium* detection derived from nested-PCR products targeting the 1325 bp (first round) and 819-835 bp (second round) of the 18S rRNA.

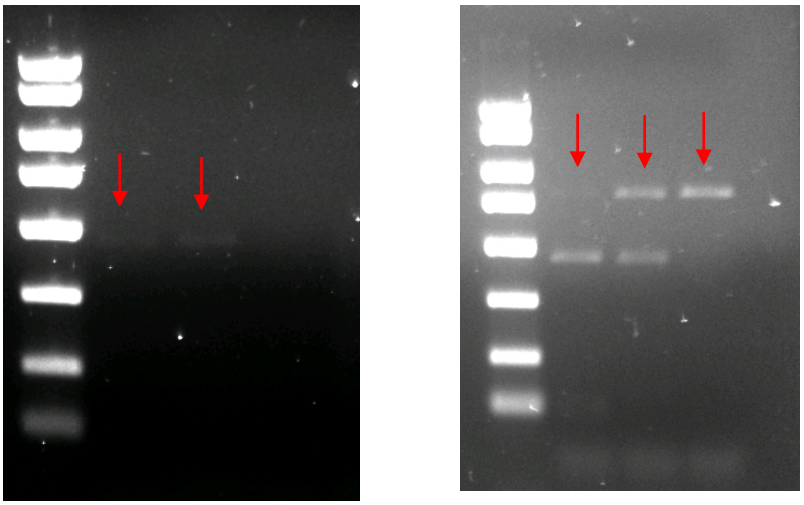
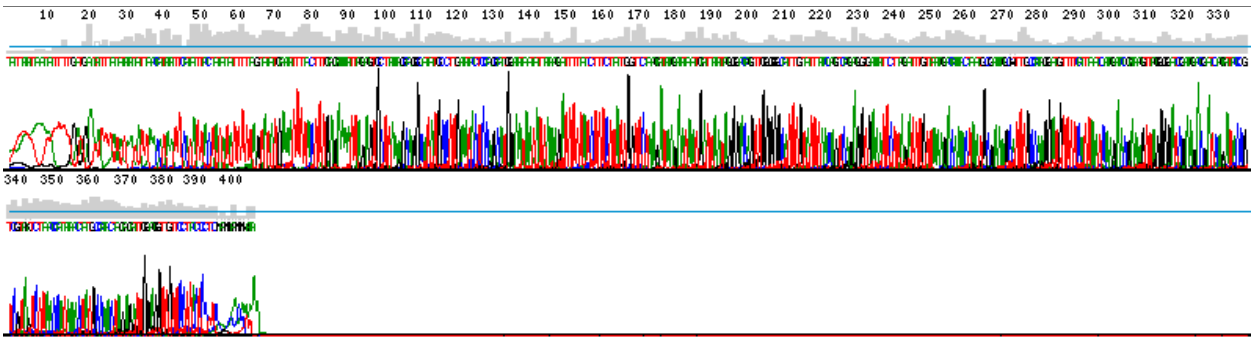
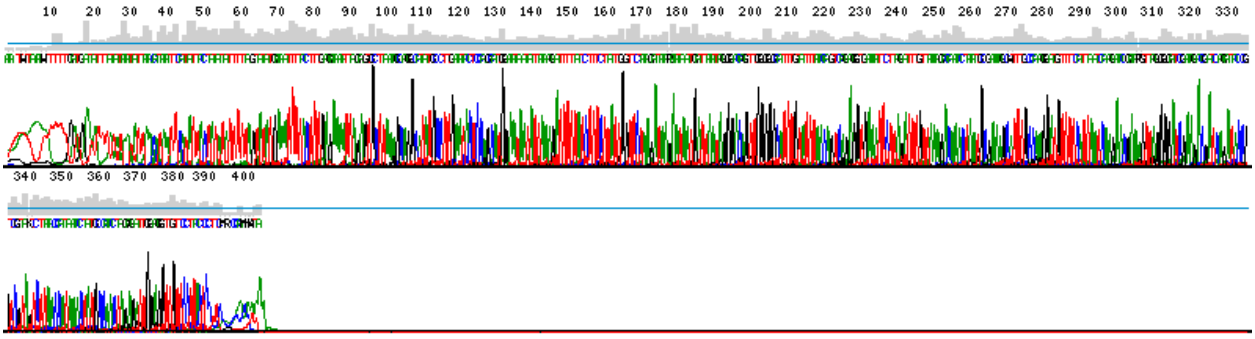


Figure D.III.: Electrophoresis images confirming DNA used for *Cryptosporidium* Positive Controls. PCR products targeting (a) 435 bp and (b) 1325 bp (first round) and 819-835 (second round) of 18S rRNA.

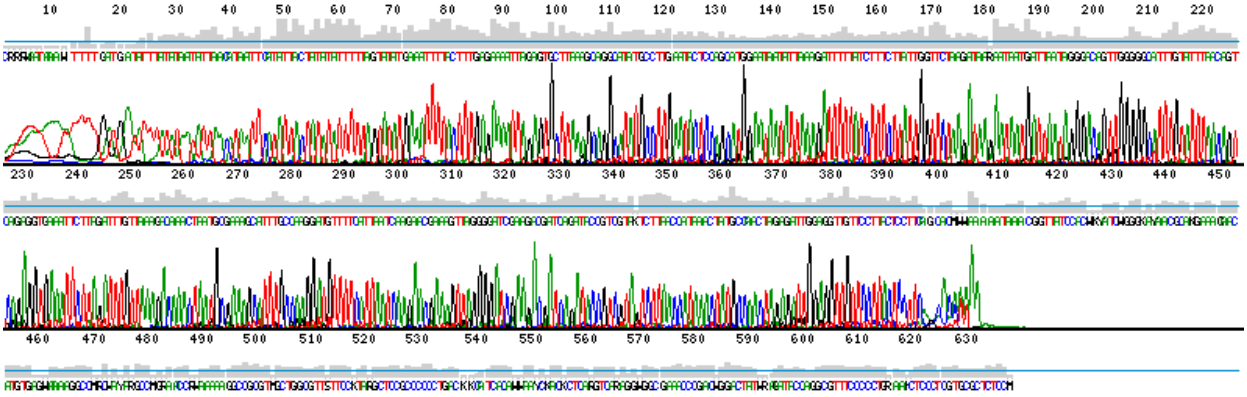
Appendix E: Chromatogram Readings of 18S rRNA Multiple Sequence Alignments



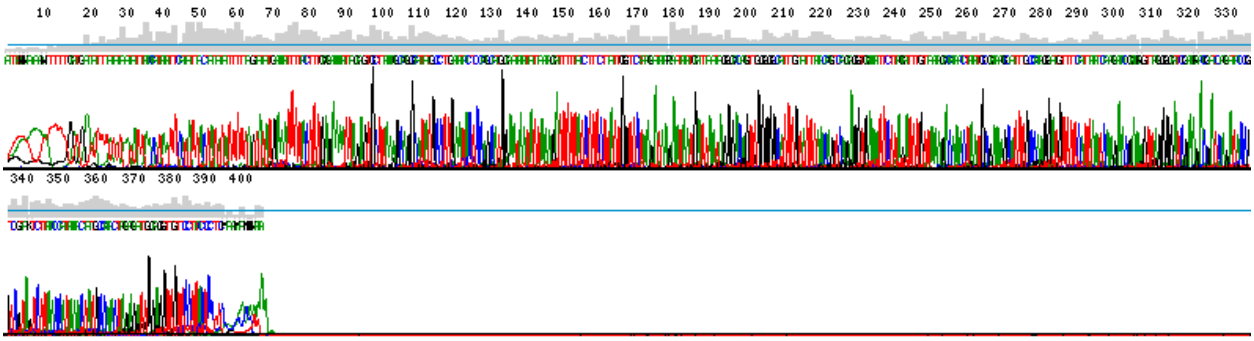
Biofilm Sample: C2Bio



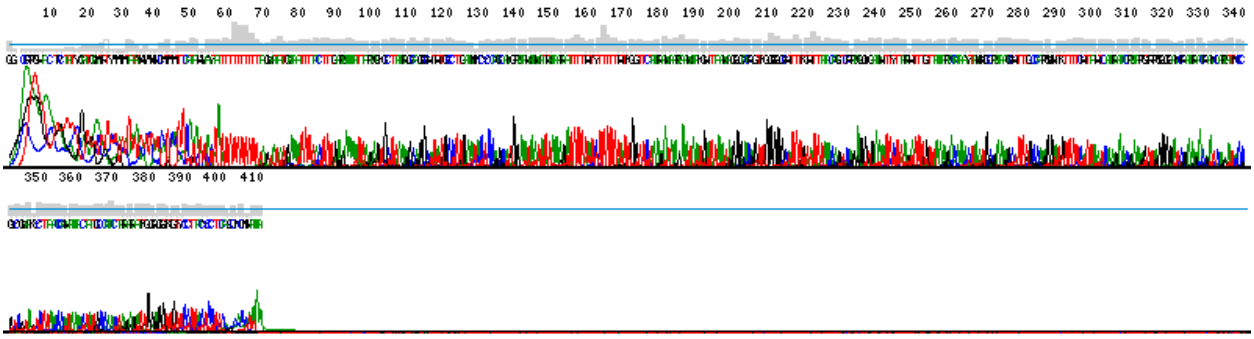
Biofilm Sample: D1Bio



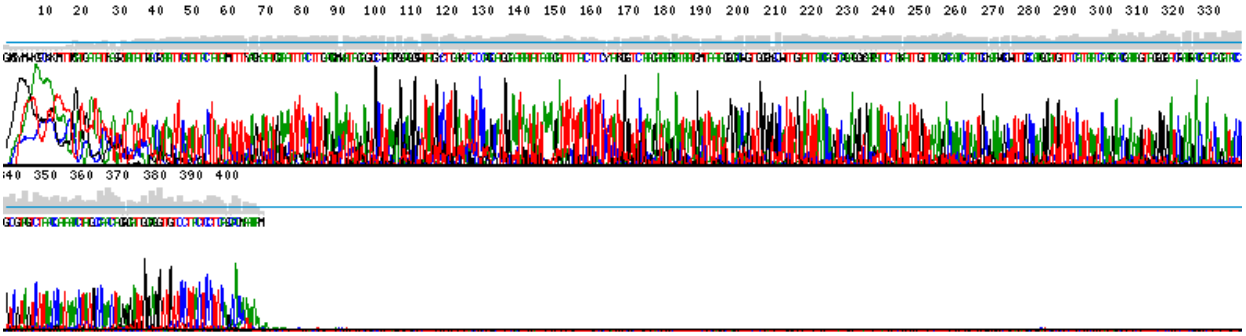
Biofilm Sample: D3Bio



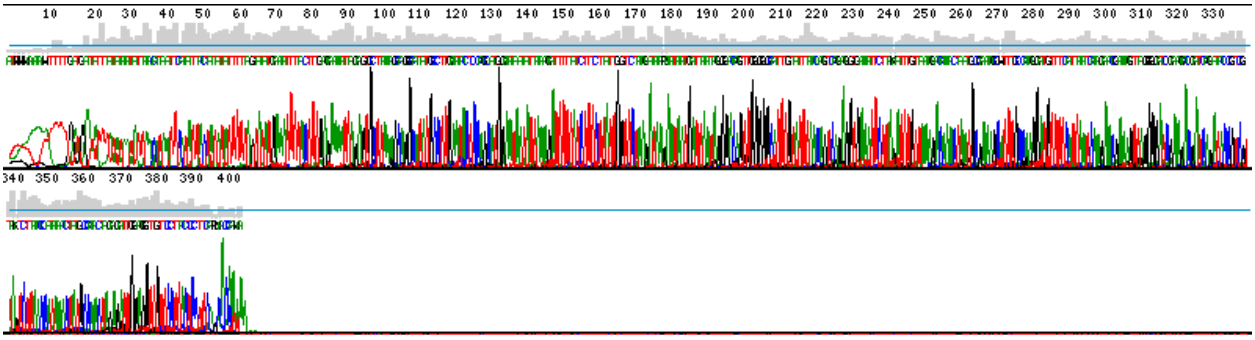
Biofilm Sample: D4Bio



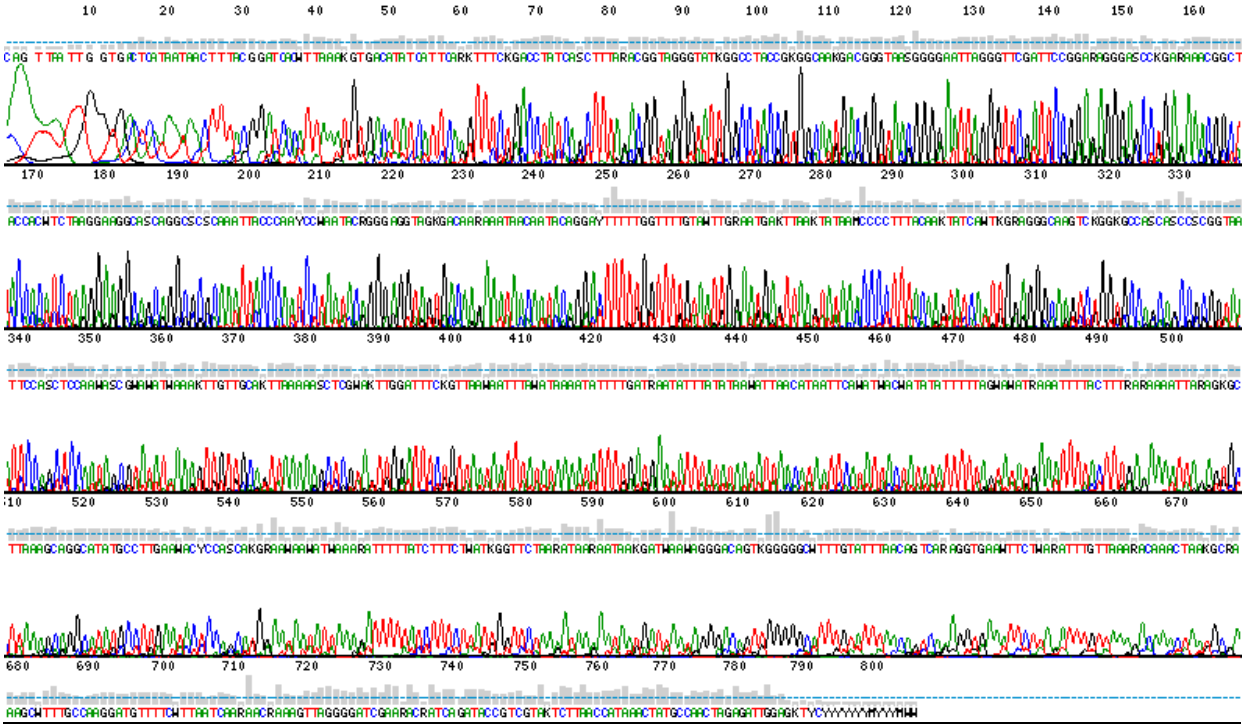
Cattle Sample: C8Cw



Cattle Sample: 34DaCw



Cattle Sample: 53DaCw



Cattle Sample: 105Cw

Figure E.I.: Chromatogram readings of 18S rRNA sequences derived from *Cryptosporidium spp.* identified in biofilm and cattle samples

Appendix F: Cost Comparison of EPA Method 1623 and the Biofilm Sampler Technique

Table F.I.: Approximate Equipment and Service Costs of *Cryptosporidium* Detection by Method 1623-Filtration/IMS/IFA

METHOD #1: EPA Method 1623- Filtration/IMS/IFA Detection										
Filter/ Capsule Equipment:										
IDEXX Filta-Max® Filter Modules- sold as 10 pack- $929.99 (part no. 98-10601-00)										$92.99
Pall Corporation EnvirochekTM HV Sampling Capsule (part no. 12099)										$141.51
Pall Corporation EnvirochekTM Sampling (part no. 12110)										$162.49
Average cost per filter/capsule:										**$132.33**
Sample Collection Equipment:										
Carboy Jug										**$25.00**
Water Pump (40-60 psi)										**$220.00***
Water Elution and Microscopic Services (quote provided by Hyperion Research Ltd., Medicine Hat, AB):										**$685.00**
Includes: elution via Filta-Max® system; IMS; IFA, DAPI, DIC microscopy										
Equipment and Service Costs										
Expense	1 Sample	2 Samples	3 Samples	4 Samples	5 Samples	6 Samples	7 Samples	8 Samples	9 Samples	10 Samples
Equipment	$377.33	$534.66	$691.99	$849.32	$1,006.65	$1,163.98	$1,324.31	$1,481.64	$1,638.97	$1,796.30
Service	$685.00	$1,370.00	$2,055.00	$2,740.00	$3,425.00	$4,110.00	$4,795.00	$5,480.00	$6,165.00	$6,850.00
Total:	**$1,062.33**	**$1,904.66**	**$2,746.99**	**$3,589.32**	**$4,431.65**	**$5,273.98**	**$6,119.31**	**$6,961.67**	**$7,803.97**	**$8,646.30**

Table F.II.: Approximate Equipment and Service Costs of Cryptosporidium Detection by Biofilm Sampler Technique- IMS/IFA

METHOD #2: *Biofilm Sampler* Technique- IMS/IFA Detection										
Biofilm Sampler Equipment:										
Customized and Reusable *Biofilm Sampler* Apparatus										**$250.00***
8 Plastic Slides (approx. $1.00 ea.)										**$8.00**
Sample Collection Equipment:										
Whirl-Pak® Stand-Up Thio-Bag®- sold as 100 pack (cat. no. WPB01403WA)										**$77.00***
Consumables										**$10.00***
IMS Reagents, Kits and Consumables:										
DynabeadsTM anti-Cryptosporidium kit (cat. no. 73011)										**$758.40***
Dynabeads™ Rotary Mixer (cat. no. 94701)										**$706.00***
Dynabeads™ MPC™-1- Magnetic Particle Concentrator (cat. no. 12001D)										**$422.00***
Dynabeads™ MPC™-S- Magnetic Particle Concentrator (cat. no. A13346)										**$878.00***
Dynabeads™ L10 Tubes (cat. no. 74003)										**$94.75***
Consumables										**$50.00***
Microscopic (IFA) Analysis- Reagents and Equipment:										
Crypt-a-Glo™ Comprehensive Kit (cat. no. 73001)										**$610.00***
Dynabeads™ Spot-On™ Slides (cat. no. 74004)										**$155.00***
Consumables										**$20.00***
Equipment and Service Costs										
Expense	1 Sample	2 Samples	3 Samples	4 Samples	5 Samples	6 Samples	7 Samples	8 Samples	9 Samples	10 Samples
Equipment	$4,039.15	$4,047.15	$4,055.15	$4,063.15	$4,071.15	$4,079.15	$4,087.15	$4,095.15	$4,103.15	$4,111.15
Service	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Total:	**$4,039.15**	**$4,047.15**	**$4,055.15**	**$4,063.15**	**$4,071.15**	**$4,079.15**	**$4,087.15**	**$4,095.15**	**$4,103.15**	**$4,111.15**

Table F.III.: Approximate Equipment and Service Costs of Cryptosporidium Detection by Biofilm Sampler Technique- IMS/PCR

METHOD #3: *Biofilm Sampler* Technique- IMS/PCR Detection	
Biofilm Sampler Equipment:	
Customized and Reusable *Biofilm Sampler* Apparatus	**$250.00***
8 Plastic Slides (approx. $1.00 ea.)	**$8.00**
Sample Collection Equipment:	
Whirl-Pak® Stand-Up Thio-Bag®- sold as 100 pack (cat. no. WPB01403WA)	**$77.00***
Consumables	**$10.00***
IMS Reagents, Kits and Consumables:	
DynabeadsTM anti-Cryptosporidium kit (cat. no. 73011)	**$758.40***
Dynabeads™ Rotary Mixer (cat. no. 94701)	**$706.00***
Dynabeads™ MPC™-1- Magnetic Particle Concentrator (cat. no. 12001D)	**$422.00***
Dynabeads™ MPC™-S- Magnetic Particle Concentrator (cat. no. A13346)	**$878.00***
Dynabeads™ L10 Tubes (cat. no. 74003)	**$94.75***
Consumables	**$50.00***
PCR Reagents, Kits and Consumables:	
QIAamp Fast DNA Stool Mini Kit (cat. no. 51604)	**$302.00***
HotStarTaq Master Mix Kit (250 U) (cat. no. 203443)	**$259.00***
Primers (quote provided by ACGT Corp.)	**$23.73***
Consumables	**$20.00***
Electrophoresis Services (quote provided by The Hospital for Sick Children- The Centre for Applied Genomics, Toronto, ON):	
8 products ($2.00 per sample)	**$16.00**

Equipment and Service Costs

Expense	1 Sample	2 Samples	3 Samples	4 Samples	5 Samples	6 Samples	7 Samples	8 Samples	9 Samples	10 Samples
Equipment	$3,858.88	$3,866.88	$3,874.88	$3,882.88	$3,890.88	$3,898.88	$3,906.88	$3,914.88	$3,922.88	$3,930.88
Service	$16.00	$32.00	$48.00	$64.00	$80.00	$96.00	$112.00	$128.00	$144.00	$160.00
Total:	**$3,874.88**	**$3,898.88**	**$3,922.88**	**$3,946.88**	**$3,970.88**	**$3,994.88**	**$4,018.88**	**$4,042.88**	**$4,066.88**	**$4,090.88**

Table F.IV.: Cost Comparison Analysis of MEHODS 1, 2 & 3

Cost- Comparison Analysis

Method no.	1 Sample	2 Samples	3 Samples	4 Samples	5 Samples	6 Samples	7 Samples	8 Samples	9 Samples	10 Samples
METHOD #1	**$1,062.33**	**$1,904.66**	**$2,746.99**	**$3,589.32**	**$4,431.65**	**$5,273.98**	**$6,119.31**	**$6,961.67**	**$7,803.97**	**$8,646.30**
METHOD #2	**$4,039.15**	**$4,047.15**	**$4,055.15**	**$4,063.15**	**$4,071.15**	**$4,079.15**	**$4,087.15**	**$4,095.15**	**$4,103.15**	**$4,111.15**
METHOD #3	**$3,874.88**	**$3,898.88**	**$3,922.88**	**$3,946.88**	**$3,970.88**	**$3,994.88**	**$4,018.88**	**$4,042.88**	**$4,066.88**	**$4,090.88**

Explanation

METHOD #1- EPA Method 1623 (Filtration/IMS/IFA):

Least expensive when analyzing 1-4 samples; Most expensive when analyzing 5-10 samples

METHOD #2 & METHOD #3- *Biofilm Sampler* Technique (IMS/IFA & IMS/PCR):

Most expensive when analyzing 1-4 samples; Least expensive when analyzing 5-10 samples

* Cost associated with reagents, kits and consumables that can be used more than once

Notes:

i) All quotes are provided in CAD or have been converted from USD to CAD at the conversion rate on 07/14/2017

ii) Analysis does not include the costs of universal microbiology laboratory equipment (e.g., PCR machine, centrifuge, micro-pipettes, votex, glassware etc.)

iii) Consumables refer to the estimated price for disposable laboratory equipment

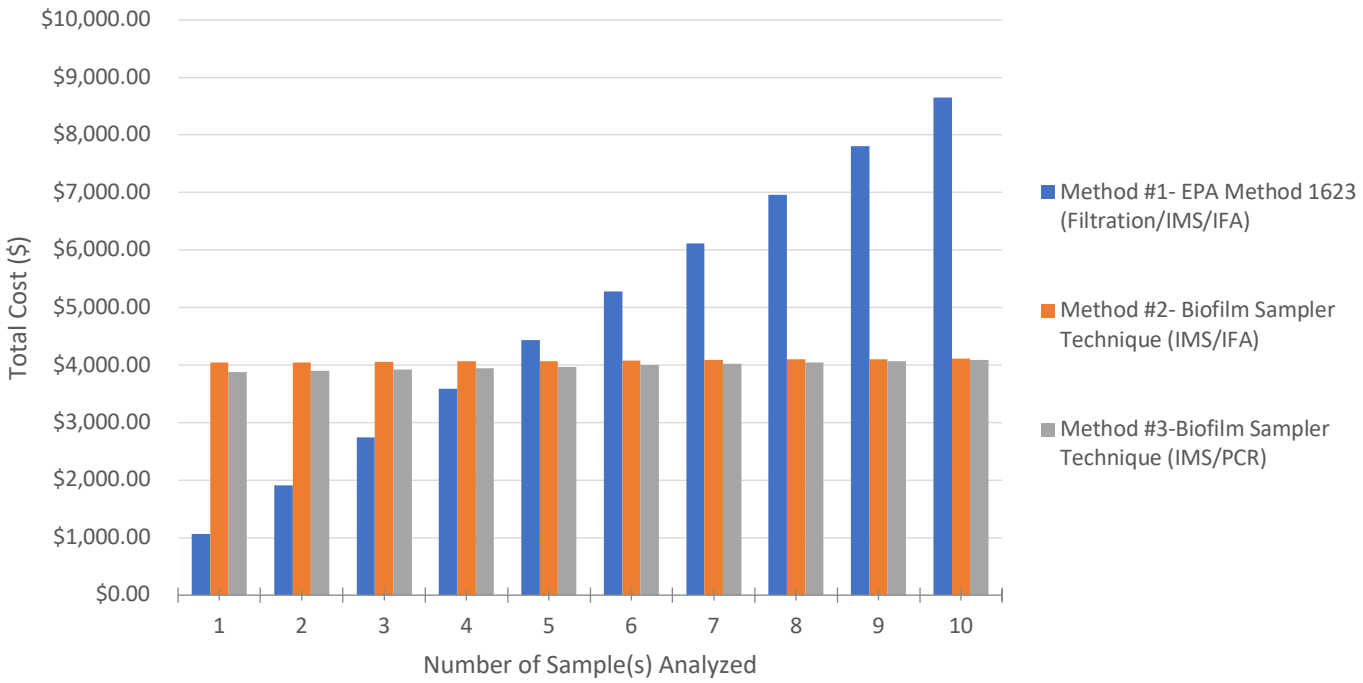


Figure F.I.: Cost-comparison analysis of EPA Method 1623 (filtration/IMS/IFA) and two versions of the *biofilm sampler* technique (IMS/IFA & IMS/PCR)

Appendix G: Photographs of Biofilm Sampling Locations and Cattle Farms

Site A (Middle Quarters)

Site B (Cataboo)

Site C (Black River City)

Site D (Lacovia)

Site E (Bartons)

Figure G.I: Photographs of biofilm sampling sites A-E. Site C highlighting the close proximity of cattle farm to the main river. Site E illustrating organic waste contamination from large-scale cultivation upstream.

Figure G.II.: Photographs of dairy and beef cattle, and rearing systems.

Appendix H: 3D Confocal Images of Biofilm Formation

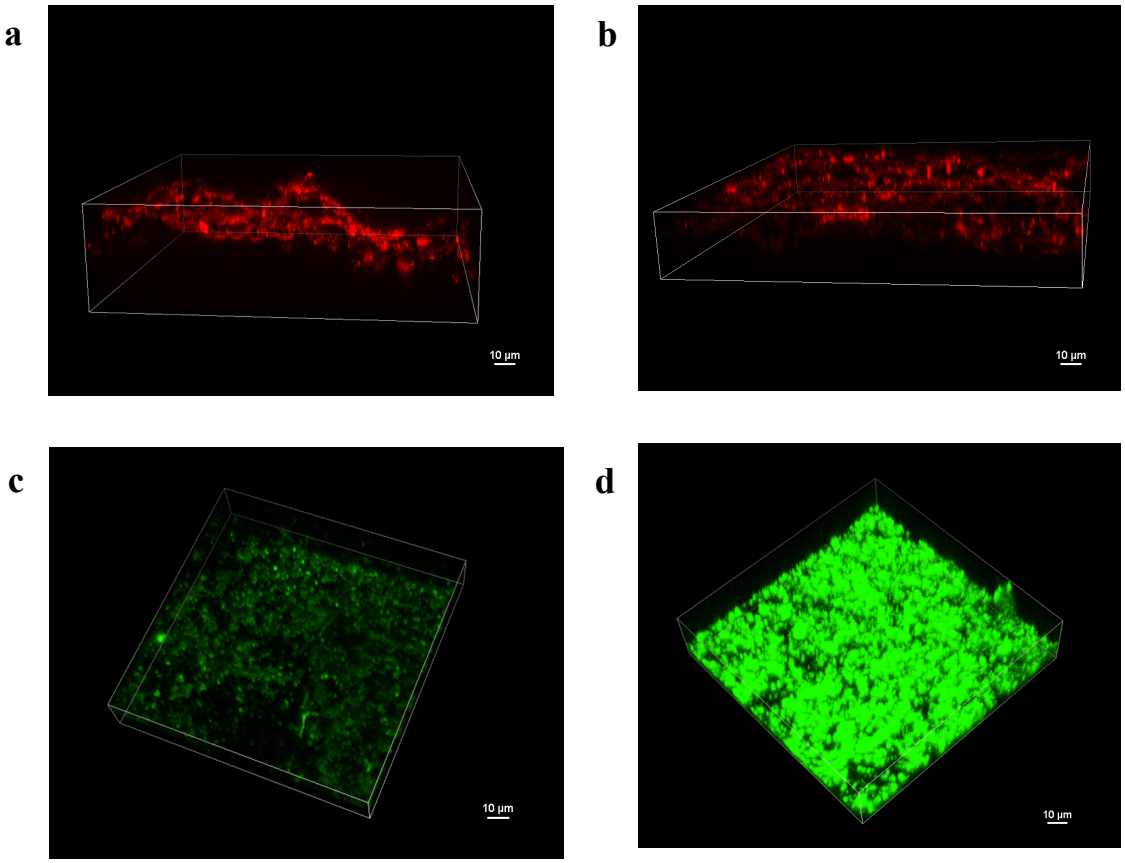


Figure H.I.: Three-dimensional, z-stack imaging of biofilm segments. Captured under confocal microscopy using Streptavidin Texas Red™ Conjugate and SYTO™ 9 Green Fluorescent Nucleic Acid Stain. Polycarbonate substrate displays an uneven/jagged formation (a, b) with a patchy surface (d). Greater microbial surface coverage was developed on the polycarbonate slide (c) compared to glass microscope slide (d). Magnification, 60X.

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