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Detection of Infectious *Cryptosporidium* in Conventionally Treated Drinking Water

Subject Area: Water Quality




Detection of Infectious *Cryptosporidium* in Conventionally Treated Drinking Water

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Detection of Infectious *Cryptosporidium* in Conventionally Treated Drinking Water

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FOREWORD

The Water Research Foundation (Foundation) is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of consultation with subscribers and drinking water professionals. Under the umbrella of a Strategic Research Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection. The Foundation also sponsors research projects through the unsolicited proposal process; the Collaborative Research, Research Applications, and Tailored Collaboration programs; and various joint research efforts with organizations such as the U.S. Environmental Protection Agency, the U.S. Bureau of Reclamation, and the Association of California Water Agencies.

This publication is a result of one of these sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals.

Projects are managed closely from their inception to the final report by the Foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The Foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, through which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver and consultants and manufacturers subscribe based on their annual billings. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The Foundation's trustees are pleased to offer this publication as a contribution toward that end.

Roy L. Wolfe, Ph.D.
Chair, Board of Trustees
Water Research Foundation

Robert C. Renner, P.E.
Executive Director
Water Research Foundation

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We are especially grateful to management and staff at all of the participating utilities that agreed to help in this project. Without their generous donation of time and supplies to filter thousands of liters of drinking water, this project would not have been possible. Their dedication to further the science of providing high quality drinking water to the public is commendable.

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EXECUTIVE SUMMARY

OBJECTIVES

The primary objective of the project was to determine the prevalence of infectious *Cryptosporidium* spp. in conventionally treated drinking water. The research covered four specific objectives: (1) assess the recovery efficiency of a modified version of U.S. Environmental Protection Agency (USEPA) Method 1623 with seeded 1,000 L finished water samples; (2) compare the sensitivity and reproducibility of three cell culture based *Cryptosporidium* infectivity assays; (3) evaluate the suitability of all methods for genotyping infectious oocysts; and (4) use the most effective method for a nationwide survey of infectious *Cryptosporidium* oocysts in large volume samples of drinking water.

BACKGROUND

Almost two decades after the Milwaukee *Cryptosporidium* incident, outbreaks of cryptosporidiosis still occur, linked to both drinking water and recreational water. Research studies report high frequencies of *Cryptosporidium* oocyst detection in untreated and finished drinking water, although monitoring programs typically demonstrate lower occurrence. Oocysts are resistant to chlorine disinfection at the concentrations typically applied during drinking water treatment. However, correctly operating treatment plants that utilize filtration usually remove oocysts from source water with high efficiency. Nevertheless, oocysts have been detected in up to 40% of treated drinking water samples at concentrations as high as 0.5 oocysts/L.

The results of plant influent monitoring under the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) will determine whether water utilities need to install additional treatment based on average *Cryptosporidium* oocyst concentrations in their source waters. Most utilities are likely to be placed in Bin 1 (<0.075 oocysts/L) requiring no additional treatment. However, there is a lot of uncertainty in estimating the contribution of drinking water to the nationwide cryptosporidiosis burden. According to the only study on the prevalence of infectious *Cryptosporidium* oocysts in conventionally filtered drinking water in the U.S., 1.4% of finished water samples contained infectious oocysts, and 27% of surface water treatment plants released infectious oocysts in their finished water at least once during the study period. The results translated to an annual cryptosporidiosis risk of 52 infections per 10,000 people, which is much higher than the 1 in 10,000 annual risk of infection goal set by the USEPA. These data indicate that public health may be compromised by municipal drinking water. Consequently, the current project aimed to assess the repeatability of the previously published study on the prevalence of infectious oocysts.

APPROACH

This study compared three assays for detecting *Cryptosporidium* spp. infections in cell culture. The assays were: (1) immunofluorescence microscopy (IFA), (2) a polymerase chain reaction targeting *Cryptosporidium* spp.-specific DNA (PCR), and (3) reverse transcriptase-PCR targeting *Cryptosporidium* spp.-specific messenger RNA (RT-PCR). Human cell monolayers (HCT-8 cells), grown in either 8-well chamber slides or 96-well plates, were inoculated with a variety of

oocysts to assess assay performance. Method evaluation included live oocysts enumerated by flow cytometry, blind-spiked samples, and oocysts that were inactivated by heat, gamma-irradiation, and ultraviolet radiation (UV). Factors used to determine the most effective method were sensitivity, intra- and inter-laboratory reproducibility, frequency of false-positives, robustness, and ease of use.

The survey of large volume samples from 14 drinking water treatment plants across the U.S (average volume = 943 L) used the most effective of the three detection assays to assess the prevalence of infectious oocysts. Sample collection, filtration through Envirochek HV capsules, elution, and immunomagnetic purification followed a modified version of USEPA Method 1623. The modification involved soaking the filter in 5% sodium hexametaphosphate prior to eluting oocysts. Recovery efficiencies were evaluated with 1,000 L matrix samples spiked with freshly shed oocysts and gamma-irradiated ColorSeed oocysts. Two laboratories performed infectivity assays for most of the finished water samples.

Infections detected by PCR and RT-PCR could be genotyped directly by sequencing primary amplification products or by amplifying and sequencing secondary target genes. In addition, the project developed a method for genotyping infections detected by IFA. The method involved lysing a focus of life stages on the IFA slide and then removing the lysate to a tube for subsequent DNA extraction, PCR, and sequencing.

RESULTS AND CONCLUSIONS

All assays detected infection of cell cultures with low numbers of flow-cytometry enumerated oocysts, including infection with a single oocyst. Based on both qualitative and quantitative comparisons, the cell culture (CC)-IFA method was selected as most effective for assessing the occurrence of infectious oocysts in finished drinking water. It consistently detected infections with three oocysts or less, generated few false-positives (all of which could be discounted by an experienced microscopist), was reproducible and relatively simple to perform. Applying the entire CC-IFA-genotyping method to naturally occurring infectious oocysts in wastewater demonstrated its suitability for environmental monitoring.

Fourteen treatment plants across the U.S. participated in the survey of infectious oocysts in finished water with sample volumes ranging from 83.5 to 2,282 L and an average of 943 L. The survey analyzed 370 samples totaling 349,053 L of treated drinking water. The volume of water filtered for each sample depended on water quality characteristics, the amount of water passing through the filter before it clogged, problems with individual filtration rigs (e.g., inadequate pressure or flow control), or operational issues at the treatment plant. Nevertheless, 90% of samples were >600 L and 82% were >900 L. Most plants provided monthly or biweekly samples for two years, although some of the plants only collected samples for part of each year.

The average recovery efficiency for 1,000 L samples of finished drinking water spiked with gamma-irradiated EasySeed oocysts and analyzed by both laboratories was 71% (n=10). The average recovery efficiency for samples from all participating utilities spiked with ColorSeed oocysts was 42% (n=45).

None of the 370 finished water samples produced infections that were detected by the CC-IFA assay. Control infections and matrix spike samples demonstrated that oocyst recovery procedures and the infectivity assay performed as expected. Based on a previously published risk assessment calculation and a total analyzed volume of 349,053 L, the lack of positives in the current study translates to an annual risk of less than one infection per 10,000 people.

The specific conclusions from this project were:

1. Infectious oocysts were not detected in 349,053 L of finished drinking water from the 14 treatment plants participating in the study.
2. The annual risk of infection for the populations served by these treatment plants, based on zero detects and the total volume of water analyzed, was <1 in 10,000.
3. Cell culture-based detection assays are sufficiently mature and standardized to be used for assessing the infectivity of *C. parvum* and *C. hominis* oocysts in finished drinking water.
4. The cell culture assay detected infection with *C. parvum, C. hominis,* and *C. meleagridis* but not *C. andersoni* or *C. muris*.
5. Oocysts can be recovered from large volumes (≥1,000 L) of finished water using a minor modification of USEPA Method 1623 and applied to cell monolayers to assess their infectivity.
6. Comparing three infectivity detection assays demonstrated the superiority of IFA over PCR and RT-PCR, based on qualitative and quantitative measures of performance.
7. Genotyping can be incorporated into non-molecular methods of infectivity detection methods such as cell culture-IFA, so that infectious oocysts can be identified to the species and sub-species level.
8. The entire method consisting of oocyst recovery by a modified Method 1623, inoculating HCT-8 cells, detecting infection by IFA, and genotyping, can be applied to naturally occurring oocysts in environmental water samples.

APPLICATIONS AND RECOMMENDATIONS

Cryptosporidium spp. oocysts are resistant to chlorine disinfection at the concentrations typically applied in drinking water treatment plants. Although correctly operating treatment plants that use filtration usually remove oocysts from water with high efficiency, low levels of *Cryptosporidium* oocysts occur in finished drinking water. Current monitoring programs using Method 1623 will provide oocyst occurrence data for untreated source waters but will not provide information on oocysts in finished water or assess the infectivity of detected oocysts. Therefore, it will still be difficult to assess the actual public health risk posed by *Cryptosporidium* in drinking water.

This project applied a standardized cell culture assay to environmentally-relevant low numbers of oocysts recovered from large volumes of finished water using a modified version of Method 1623. The cell culture method involved incubating inoculated HCT-8 cells in 8-well chamber slides at 37°C for 64–72 hours, staining with anti-sporozoite antibody and a FITC-labeled secondary antibody, and enumerating infections by epifluorescence microscopy. Widespread application of this method to finished water will allow a more accurate assessment, with increased confidence, of the public health significance of *Cryptosporidium* oocysts in drinking water.

The inter-laboratory method comparisons demonstrated that the CC-IFA method is suitable for monitoring infectious *Cryptosporidium* in finished water. Information on the relative sensitivity of the methods and their rates of false-positive detections will allow other investigators and utilities to make a more informed decision when selecting a method for either routine monitoring or stand-alone research studies.

The project highlighted the difficulty in applying a non-compliance microbiological method when the results could have adverse legal, operational, public health, and public relations consequences for participating utilities. Many utilities were reluctant to participate because of concerns over the possible consequences of detecting infectious oocysts in their finished drinking water. Consequently, the majority of utilities participating in this study had low levels of *Cryptosporidium* in their source waters and so detecting infectious *Cryptosporidium* in their finished water was unlikely.

Since a broader range of utilities may need to be surveyed for the presence of infectious *Cryptosporidium* oocysts, the second round of *Cryptosporidium* monitoring under the LT2ESWTR should include infectivity analyses on finished water. Mandatory infectivity analyses would not be practical for all utilities but a subset of utilities could be monitored on a relatively frequent basis. The cell culture method is sufficiently developed and standardized that the laboratory capacity could be readily built within the regulatory timeframe. Options for implementing cell culture-based infectivity monitoring include: (1) on-site cell culture facilities at utility laboratories; (2) purchasing ready to use cell monolayers from a commercial supplier and then performing oocyst recovery and infectivity assay procedures in-house; and (3) shipping recovered oocysts to a centralized cell culture testing facility.

Specific project recommendations include:

1. Implement monitoring for infectious *Cryptosporidium* oocysts in finished water using a standardized cell culture assay. These assays may be carried out using in-house facilities or contract laboratories.
2. Conduct follow-up studies that include state public health professionals and federal regulators as part of the project team. This expanded team may help to reduce the reluctance of utilities to participate.
3. Focus future surveys on Bin 2 or higher utilities rather than attempting to capture a national average risk of infection. Bin 2 and higher utilities represent an increased risk of infection compared to the majority of plants, which will be classified as Bin 1. Surveys could include intensive sampling of a few plants over an extended period.
4. Optimize the *Cryptosporidium* cell culture method, to increase proportional infectivity, which will increase the likelihood of detecting infection with a single oocyst.
5. Assess the range of *Cryptosporidium* species and genotypes that can infect HCT-8 cells and the specificity of the anti-sporozoite antibody to infectious stages of species other than *C. parvum, C. hominis,* and *C. meleagridis*.

PARTICIPANTS

This project could not have been possible without the generous support and participation of the 14 anonymous utilities that provided filtered water samples throughout the study.

CHAPTER 1
INTRODUCTION

STATEMENT OF PROBLEM

Background

Protozoan parasites of the *Cryptosporidium* genus are common in many animal species including mammals, marsupials, reptiles, birds, and fish (Fayer 2008; Fayer et al. 2000). There have been many outbreaks of cryptosporidiosis associated with either drinking water or recreational use of water (Fayer et al. 2000). The largest waterborne outbreak to date occurred in 1993 in Milwaukee with estimates of the affected population ranging from 15,000 to 400,000 individuals (Hunter and Syed 2001; MacKenzie et al. 1994). The continued detection of *Cryptosporidium* oocysts in source water and treated drinking water ensures that the organism remains a significant concern for the water industry and mandated monitoring under the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) will determine whether water utilities need to install additional treatment based on the level of *Cryptosporidium* in their source water.

The genus *Cryptosporidium* contains at least 16 recognized species that infect a variety of vertebrates. The organisms are coccidian parasites placed within the Phylum Apicomplexa (Fayer 2008). Although *Cryptosporidium parvum* and *Cryptosporidium hominis* are the species most often isolated from humans, other species have also been detected in immune-compromised individuals. These include *C. canis*, *C. felis*, *C. meleagridis*, and *C. muris* (Fayer et al. 2001; Gatei et al. 2002; Morgan et al. 2000; Morgan-Ryan et al. 2002; Pedraza-Diaz et al. 2001; Pieniazek et al. 1999; Xiao et al. 2001). However, most cases of human cryptosporidiosis are attributed to *C. parvum* and *C. hominis*. Infections in humans may be asymptomatic but more frequently result in a variety of self-limiting acute enteric symptoms characterized by profuse diarrhea, and persistent infection of severely immune-compromised patients can contribute to mortality. Currently there is only limited treatment available for cryptosporidiosis in immune-competent individuals (Anderson and Curran 2007).

Cryptosporidium in Water

A review of eight studies assessing the occurrence of *Cryptosporidium* spp. in untreated source waters in the United States demonstrated that the average proportion of river, lake, and well water samples that were contaminated with oocysts ranged from 9 to 100% (Rose et al. 1997). Water samples that were impacted by domestic and agricultural waste had oocyst concentrations as high as 5,800/L (Madore et al. 1987). A large survey of North America spanning 1988–1993 reported that 60.2% of samples (N=347) were positive for *Cryptosporidium* oocysts (LeChevallier and Norton 1995). A similar study in Canada demonstrated lower levels of contamination with oocysts detected in 6.1%, 4.5%, and 3.5% of raw sewage, raw water, and treated drinking water, respectively (Wallis et al. 1996). Additional studies have reported the occurrence of oocysts in 6% of stream samples in Wisconsin (Archer et al., 1995), 63% of river samples in Pennsylvania (States et al. 1997), and 13% of surface waters in New Zealand (Ionas et al. 1998). A large watershed survey conducted by the Metropolitan Water District of Southern California (MWDSC) detected

oocysts in 11% of samples (N=189) and 24% of first flush samples (N=34) with extrapolated oocyst concentrations up to 417/L following storm events (Ferguson et al. 1998). The Information Collection Rule (ICR) survey of 5,838 untreated source waters throughout the U.S. reported an average occurrence of 6.8% with a mean concentration of 0.067 oocysts/L (Messner and Wolpert 2003).

A compilation of genotyping data from 22 waterborne outbreaks of cryptosporidiosis demonstrated that 67% were caused by *C. hominis* while *C. parvum* was the causative agent in the remaining 33% (McLauchlin et al. 2000; Sulaiman et al. 1998). Out of a total of 29 storm water samples analyzed by a PCR-restriction fragment length polymorphism analysis targeting the SSU rRNA gene, 93% were positive for *Cryptosporidium* with 12 different genotypes represented (Xiao et al. 2000). None of the detected genotypes matched those typically found in human, farm animal, or domestic animal samples. However, four were identical or closely related to *C. baileyi*, and *Cryptosporidium* genotypes from opossums and snakes indicating that wildlife was the primary source of oocyst contamination of surface water during storms. The same method was also used to analyze untreated surface water and wastewater samples. *Cryptosporidium* was detected in 45.5% of surface water samples (N=55) and 24.5% of raw wastewater samples (N=49; Xiao et al. 2001). The predominant genotypes in surface water matched the profiles of *C. parvum* and *C. hominis* while *C. andersoni* was most commonly detected in wastewater.

While oocysts are resistant to chlorine disinfection at the concentrations typically applied during drinking water treatment, correctly operating treatment plants that utilize filtration usually remove oocysts from source water with high efficiency. However, oocysts have been detected in 3.8–40% of treated drinking water samples at concentrations up to 48 oocysts/100 L (Rose et al. 1997). A survey of treatment plants in Wisconsin detected oocysts in 4.2% (N=72) of finished water samples (Archer et al. 1995). In addition, a study utilizing cell culture to assess infectivity reported that 26.8% of surface water treatment plants (N=82) were releasing infectious oocysts in their finished water (Aboytes et al. 2004). Overall, this latter study found that 1.4% of treated drinking water samples (N=1,690) contained infectious *Cryptosporidium* oocysts but in all cases the follow-up repeat sample was negative. This detection rate translated into a calculated annual risk of infection of 1 in 193 or 52 infections in 10,000 people, far exceeding the U.S. Environmental Protection Agency's (USEPA) 1 in 10,000 risk goal. However, further surveys need to be conducted on the occurrence of infectious *Cryptosporidium* in treated drinking water to corroborate this earlier study.

Assessing Viability and Infectivity of *Cryptosporidium*

Cryptosporidium spp. are obligate, intracellular parasites that infect the epithelial cells lining the luminal surfaces of the digestive and respiratory tracts of a wide variety of animal hosts. Viability has been measured with vital dyes (Korich et al. 1990) and excystation (Robertson et al. 1993) with mixed results. However, an oocyst may be viable but not infectious. Therefore, to determine the actual public health risk posed by waterborne *Cryptosporidium*, the infectivity of oocysts must be determined. Infectivity is assessed using human volunteers, animal models, or in-vitro cell culture. The description of complete life-cycle development and de-novo oocyst production in cell-free media (Hijjawi et al. 2004) may require a reassessment of the basic biology of the organism, but pending further corroboration *C. parvum* is still generally considered to be a host-dependent parasite.

Table 1.1
Cell culture-based infectivity assays for *C. parvum*

Assay format	Cell line	Detection method	Reference
Thermanox coverslips	RL95-2	Giemsa staining	Rasmussen et al. 1993
Glass coverslips	MDBK	Interference microscopy	Upton et al. 1994
Glass coverslips	BFTE	Microscopy	Forney et al. 1996
Permeable membranes	Caco-2	Transmonolayer resistance	Griffiths et al. 1994
96-well plates	MDCK	Chemiluminescence immunoassay	You et al. 1996
96-well plates	HCT-8	ELISA	Woods et al. 1996
96-well plates	HCT-8	PCR on DNA (hsp70)	Di Giovanni et al. 1999
24-well plates	BS-C-1	PCR on DNA	Deng and Cliver 1998
24-well plates	HCT-8	PCR on DNA(18S rRNA)	Keegan et al. 2003
Chamber slides	MDCK	Immunofluorescence	Arrowood et al. 1994
Chamber slides	HCT-8	Immunofluorescence	Slifko et al. 1997,1999
Chamber slides	Caco-2	RT-PCR on mRNA (hsp70)	Rochelle et al. 1997, 2002

From the perspective of data accuracy and extrapolation, the ideal model for assessing the infectivity of a human pathogen would be a representative selection of *Homo sapiens*. However, human infectivity assays are not practical for use on a routine basis due to the difficulty in obtaining a sufficiently large cohort size, ethical concerns surrounding human testing, and the potentially serious and long-term health effects for study participants. Artificial infection of calves is the most common method for propagating oocysts of *C. parvum* since the parasite undergoes considerable amplification in newborn cattle. A wide variety of animals, including hamsters, rats, macaques, pigs, lambs, and opossums have been used for *C. parvum* infectivity assays, but the most commonly used animal models are various strains of adult and suckling mice. Although infectivity in mice was considered to be the gold standard for measuring *C. parvum* infectivity, there are drawbacks to the approach. The use of animals in scientific research raises ethical concerns, and animal-based assays are expensive, time-consuming and have significant hidden costs, such as the maintenance of accredited facilities and license fees. A further disadvantage has been the inability of mouse infectivity models, including GKO mice, to support infection of *C. hominis* (Peng et al. 1997; Widmer et al. 2000). *C. hominis* has been propagated to a limited extent in gnotobiotic piglets and has demonstrated infection in calves (Akiyoshi et al. 2002). However, no routine animal model is yet available for testing the infectivity or response to disinfectants of *C. hominis*.

At least 21 cell lines support *C. parvum* infection and infectivity assays have been developed using a variety of cell lines, assay formats, and detection methods (Table 1.1). The ELISA and chemiluminescence immunoassays in 96-well formats have been useful for large scale screening of potential anticryptosporidial agents (Woods et al. 1995; You et al. 1996). The microscopic detection methods provide readily enumerated infectivity results because the developmental stages are visualized. However, such procedures can be time consuming. Molecular-based infection detection methods utilizing PCR to amplify either DNA or mRNA have also been developed (DiGiovanni et al. 1999; Rochelle et al. 1997; Rochelle et al. 2002). These techniques are highly specific and sensitive and can be used to screen a large number of samples. An assay using RT-PCR to amplify *C. parvum*-specific mRNA from a region of the 70 kDa heat shock protein gene (hsp70) was used to detect infection in Caco-2 and HCT-8 cells with as few as 10 oocysts (Rochelle et al. 1997). RT-PCR detection of infection on HCT-8 cells was used to demonstrate that oocysts recovered from environmental water samples by immunomagnetic separation and by USEPA Method

Table 1.2
Prevalence of infectious *Cryptosporidium* spp. in water

Type of water	Number of samples	Positive	Reference
Source water	560	3.9%	LeChevallier et al. 2003
Source water	122	4.9%	Di Giovanni et al. 1999
Filter backwash water	121	7.4%	Di Giovanni et al. 1999
Raw wastewater	18	33%	Gennaccaro et al. 2003
Disinfected reclaimed effluent	15	40%	Gennaccaro et al. 2003
Finished drinking water	1,690	1.4%	Aboytes et al. 2004

1622 retained their infectivity (Rochelle et al., 1999). The assay was also used to measure the efficiency of UV inactivation of *C. parvum* (Mofidi et al. 2001) and generated inactivation results that showed very close agreement to published mouse-derived data. In extensive evaluations with five isolates of *C. parvum* the assay was equivalent to the widely used CD-1/ICR mouse assay for measuring the infectivity of untreated *C. parvum* oocysts (Rochelle et al. 2002). In addition, the assay was used to demonstrate that HCT-8 cells support infection by *C. hominis* (Rochelle et al. 2002). Also, using HCT-8 cells and PCR detection targeting *C. parvum* hsp70 DNA, 4.9% of raw water samples and 7.4% of filter backwash samples contained infectious *C. parvum* (Di Giovanni et al. 1999). The sensitivity of this assay was less than five infectious oocysts. The same method also detected infectious oocysts in 3.9% of untreated source water samples (N=560, LeChevallier et al. 2003). A sensitivity of a single infectious oocyst was reported for an assay using IFA to detect infection in HCT-8 cells (Slifko et al. 1997, 1999). A modification of the IFA-MPN method detected infectious *Cryptosporidium* in 40–50% of effluent samples from water reclamation facilities (Gennaccaro et al. 2003; Quintero-Betancourt et al. 2003). Studies that have examined the prevalence of infectious *Cryptosporidium* spp. in water are summarized in Table 1.2.

Widespread application of cell culture-based infectivity assays for *Cryptosporidium* (Table 1.3) demonstrates that the method has been accepted as a reliable tool by many within the water industry. However, there has been relatively little standardization of assay conditions or procedures. In a comparison of a variety of cell lines for supporting in-vitro growth of *C. parvum*, infection in HCT-8 cells generated approximately twice as many intracellular life cycle stages compared to MDBK, MDCK, or Caco-2 cells (Upton et al. 1994). However, other investigators reported that there was no difference in the level of infection supported by Caco-2, HCT-8 or HT29 cell lines (Maillot et al. 1997) or when comparing HCT-8 and MDCK cells (You et al. 1996). Nevertheless, HCT-8 is the most widely used cell line due to its relatively easy maintenance and sensitivity to infection. Although the majority of in-vitro infections have been with *C. parvum* or *C. hominis*, other species have also been studied. For example, MDBK cells supported infection with *C. meleagridis* (Akiyoshi et al. 2003).

Different cell lines require particular types of media and additives for growth, although optimum growth conditions for the host cells may not be conducive to maximum parasite development. Standard cell culture media contain all of the essential nutrients for cell growth and are typically supplemented with fetal bovine serum (FBS) at concentrations ranging from 5% to 20%. A variety of media additives may also be added depending on the application and the preference or experience of the researchers. These include antibiotics to suppress growth of bacterial and fungal contaminants (typically penicillin, streptomycin, kanamycin, and amphotericin), HEPES buffer, glutamine, glucose, vitamins, and insulin. Detailed reviews of in-vitro cell culture approaches

Table 1.3
Use of cell culture to assess *Cryptosporidium* viability

Detection method	Application	Pathogen	Reference
CC-RT-PCR*	UV disinfection	*C. hominis*	Johnson et al. 2005
CC-RT-PCR	UV disinfection	*C. parvum*	Rochelle et al. 2004
CC-qPCR†	Effect of drug treatment	*C. parvum*	Shahiduzzaman et al. 2009
CC-qPCR	Quantitation of infectivity	*C. parvum*	Di Giovanni et al. 2005
CC-qPCR	UV, ozone, mixed oxidant, and chlorine disinfection	*C. parvum*	Keegan et al. 2003
CC-qPCR	Effect of drug treatment	*C. parvum*	MacDonald et al. 2002
CC-IFA‡	Effect of temperature on survival of oocysts in source water	*C. parvum*	Ives et al. 2007
CC-IFA	UV disinfection	*C. parvum*	Entrala et al. 2007

*Cell culture-reverse transcriptase-polymerase chain reaction.
†Cell culture-quantitative polymerase chain reaction.
‡Cell culture-immunofluorescence assay.

and media formulations have been published previously (Arrowood 2002; Rochelle and De Leon 2001; Upton 1997).

PROJECT OBJECTIVES

The overall project objective was to determine the prevalence of infectious *Cryptosporidium* oocysts in conventionally treated drinking water. Phase 1 tested different cell infectivity methods to determine which method best met the criteria for sensitivity of detection, detection of multiple isolates, and no or minimal false-positives and false-negatives. Phase 2 involved analyzing samples from selected drinking water utilities to assess the prevalence of infectious *Cryptosporidium* in finished drinking water. Positive samples would be genotyped to determine the most likely source of contamination (human, domestic animal, or wildlife). The specific objectives of this project were:

1. Assess the recovery efficiency of a modified version of USEPA Method 1623 with seeded 1,000 L finished water samples.
2. Compare the sensitivity and reproducibility of three cell culture based *Cryptosporidium* infectivity assays.
3. Evaluate the ability of all assays to incorporate infectious oocyst genotyping.
4. Use the most appropriate method for a nationwide survey of infectious *Cryptosporidium* oocysts in large volume samples of drinking water (~1,000 L) from treatment plants.

CHAPTER 2
EVALUATION AND COMPARISON OF CELL CULTURE METHODS

INTRODUCTION

Although many cell culture-based methods have been developed for assessing the infectivity of *Cryptosporidium* spp. oocysts, there has been no rigorous comparison of methods. Such a comparison is necessary to evaluate the sensitivity of the assays, and the frequency of false-positive and false-negative results. If a cell culture assay is used to assess the prevalence of infectious *Cryptosporidium* spp. in finished drinking water, and the resulting data are used to make operational, treatment, or regulatory decisions, it is imperative that the method used demonstrates high sensitivity with no or minimal risk of false-negative or false-positives. Therefore, this project was conducted in two phases.

In Phase 1, the three most commonly used cell culture assays were compared in two laboratories (MWDSC and AgriLife El Paso) with oocysts subjected to a variety of conditions. The three assays all used the human ileocecal HCT-8 cell line but differed primarily in the methods used to detect infections in cell monolayers. Following inoculation and incubation, the infection detection methods were:

- Immunofluorescence assay (IFA) involving antibody staining and microscopy.
- Reverse transcriptase polymerase chain reaction (RT-PCR) on extracted RNA.
- Polymerase chain reaction (PCR) on extracted DNA.

The assays were evaluated with low doses of viable oocysts (enumerated by flow cytometry) to determine sensitivity and with oocysts exposed to a variety of disinfectants. In addition, the methods were evaluated with two isolates of *C. parvum*, (Iowa and Moredun isolates), and a *C. hominis* isolate. The feasibility and practicality of applying genotyping techniques was also evaluated for each method. The most appropriate method was then selected to conduct a survey of finished water.

MATERIALS AND METHODS

Cryptosporidium spp. Oocysts

Source, Propagation, and Purification of Oocysts

A variety of isolates was used throughout this project (Table 2.1). Oocysts from a variety of sources were evaluated using the cell culture/RT-PCR method at MWDSC. Bovine-propagated and mouse-propagated *C. parvum* oocysts (Iowa isolate) were obtained from Waterborne, Inc. (New Orleans, LA). Bovine propagated Iowa oocysts were also obtained from the University of Arizona (Tucson, AZ) and Bunch Grass Farms (Deary, ID; previously called Pleasant Hill Farms) and the *C. parvum* Moredun isolate, propagated in sheep, was provided by Steve Wright (Moredun Institute, Penicuik, Scotland). EasySeed oocysts (BTF, Australia) were used for spiking untreated and finished drinking water samples to determine recovery efficiencies. Each EasySeed tube

Table 2.1
***Cryptosporidium* spp. isolates**

Organism	Isolate	Original host	Propagation host	Source	Reference
C. parvum[*]	Iowa	Cow	Cow	EasySeed; BTF Australia	
C. parvum	Iowa	Cow	Cow	Sterling Parasitology Laboratory Tuscon, AZ	Korich et al. 1990
C. parvum	Iowa	Cow	Cow	Bunch Grass Farms Deary, ID	Chauret et al. 2001
C. parvum	Iowa	Cow	Cow	Waterborne, Inc. New Orleans, LA	Di Giovanni et al. 2005
C. parvum	Iowa	Cow	Mouse	Waterborne, Inc. New Orleans, LA	
C. parvum	Moredun	Deer	Sheep	Moredun Research Institute Penicuik, Scotland	Blewett et al. 1993
C. hominis	TU728	Human	Pig	Tufts University North Grafton, MA	Widmer et al. 2000
C. andersoni		Cow	Cow	University of Calgary Alberta, Canada	Nichols et al. 2006
C. meleagridis	TU1867	Quail, pheasant		Tufts University North Grafton, MA	Akiyoshi et al. 2003
C. muris	RN66	Mouse	Mouse	Waterborne, Inc. New Orleans, LA	

*Gamma-irradiated oocysts.

contained 100 inactivated (gamma-irradiated) *Cryptosporidium* oocysts and 100 *Giardia* cysts in 1 mL of saline solution. Easy Seed is approved by the USEPA for use in regulated *Cryptosporidium* and *Giardia* testing. EasySeed oocysts were used to assess modified Method 1623 recovery efficiencies on 1,000 L samples of treated drinking water. The Iowa isolate was used to assess the sensitivity limits of the cell culture infection detection methods using flow cytometry enumerated and sorted viable oocysts. The Iowa isolate was also used to assess the performance of the infectivity assays with non-viable oocysts and oocysts exposed to disinfectants.

DNA was extracted from three lots of oocysts of the Iowa isolate to ensure that the mouse-propagated oocysts were the same as those propagated in cows, that they were consistent with the Iowa isolate, and as a preliminary test of the PCR genotyping primers. DNA was amplified using primers targeting a 60-kDa glycoprotein gene (GP60) and the 18S rRNA gene (Table 2.2), and sequenced (Laragen, Los Angeles, CA). The sequencing results confirmed that mouse- and bovine-propagated oocysts from Waterborne were the same and were consistent with the published GP60 sequence for the Iowa isolate (Figure 2.1). Also, the Moredun isolate obtained for this project contained an additional TCA repeat and a GA transition, consistent with the published sequence for this isolate. The results were confirmed by 18S rDNA sequences.

Table 2.2
PCR primers used for genotyping *Cryptosporidium*

Gene	Primer	Sequence (5'–3')	Amplicon size	Reference
GP60	primary, F	ATA GTC TCC GCT GTA TTC	902 bp*	Glaberman et al. 2002
	primary, R†	GGA AGG AAC GAT GTA TCT		
	nested, F†	TCC GCT GTA TTC TCA GCC	868 bp*	
	nested, R	GCA GAG GAA CCA GCA TC		
18S rRNA	primary, F	TTC TAG AGC TAA TAC ATG CG	1,325 bp	Xiao et al. 2001
	primary, R	CCC ATT TCC TTC GAA ACA GGA		
	nested, F	GGA AGG GTT GTA TTT ATT AGA TAA AG	~850 bp	
	nested, R	AAG GAG TAA GGA ACA ACC TCC A		

*Amplicons are 3 bp longer in the Moredun isolate due to an additional TCA repeat.
†The order (but not sequence) of these two primers was reversed in the original publication. They are presented in the correct order here.
F, Forward primer.
R, Reverse primer.

Enumeration of Oocysts

Oocysts were enumerated by well slide microscopy counts or flow cytometry. To enumerate oocysts using well slides, 10 aliquots of oocysts were placed on two-well Superstick slides (Waterborne, Inc., New Orleans, LA) and allowed to dry overnight at room temperature. The FITC-labeled anti-*Cryptosporidium* antibody (Cellabs, Australia) was applied to the wells and the slides were incubated at 37°C in a humidified chamber for 30 min. Following rinsing in PBS, slides were mounted and examined using a microscope equipped for epifluorescence with excitation and emission wavelength filters of 485/520 nm and 515–565 nm, respectively. The concentration of the stock oocysts was calculated from the mean of 10 individual counts. The mean coefficient of variation (CV) for the oocyst counts was ≤16%.

Flow cytometry enumeration of oocysts was done by the Wisconsin State Laboratory of Hygiene (WSLH). Quality assurance/quality control (QA/QC) of stock oocysts, including determination of proportional cell culture infectivity, was performed at both MWDSC and AgriLife El Paso before they were sent to WSLH for flow cytometry enumeration and sorting (see next section). WSLH sorted the oocysts into individual microcentrifuge tubes for each experiment. No further dilution of samples was done at the processing laboratories before inoculating the monolayers.

Quality Assurance/Quality Control of Oocysts

Every lot of oocysts was subjected to a rigorous QC evaluation prior to use for any infectivity experiments. This included: microscopic observation of wet mounts and Gram-stained samples; inoculation of nutrient broth, Sabaroud-dextrose plates, and m-endo plates to look for microbial contamination; FITC-antibody stained samples to determine if the oocysts appeared healthy and intact; and inoculation of RPMI-1640 cell culture medium containing antibiotics and 2% FBS to ensure that the oocyst preparation would not contaminate the cell culture. In addition, each lot of oocysts was tested for its ability to infect HCT-8 cells. Six HCT-8 monolayers in 8-well chamber

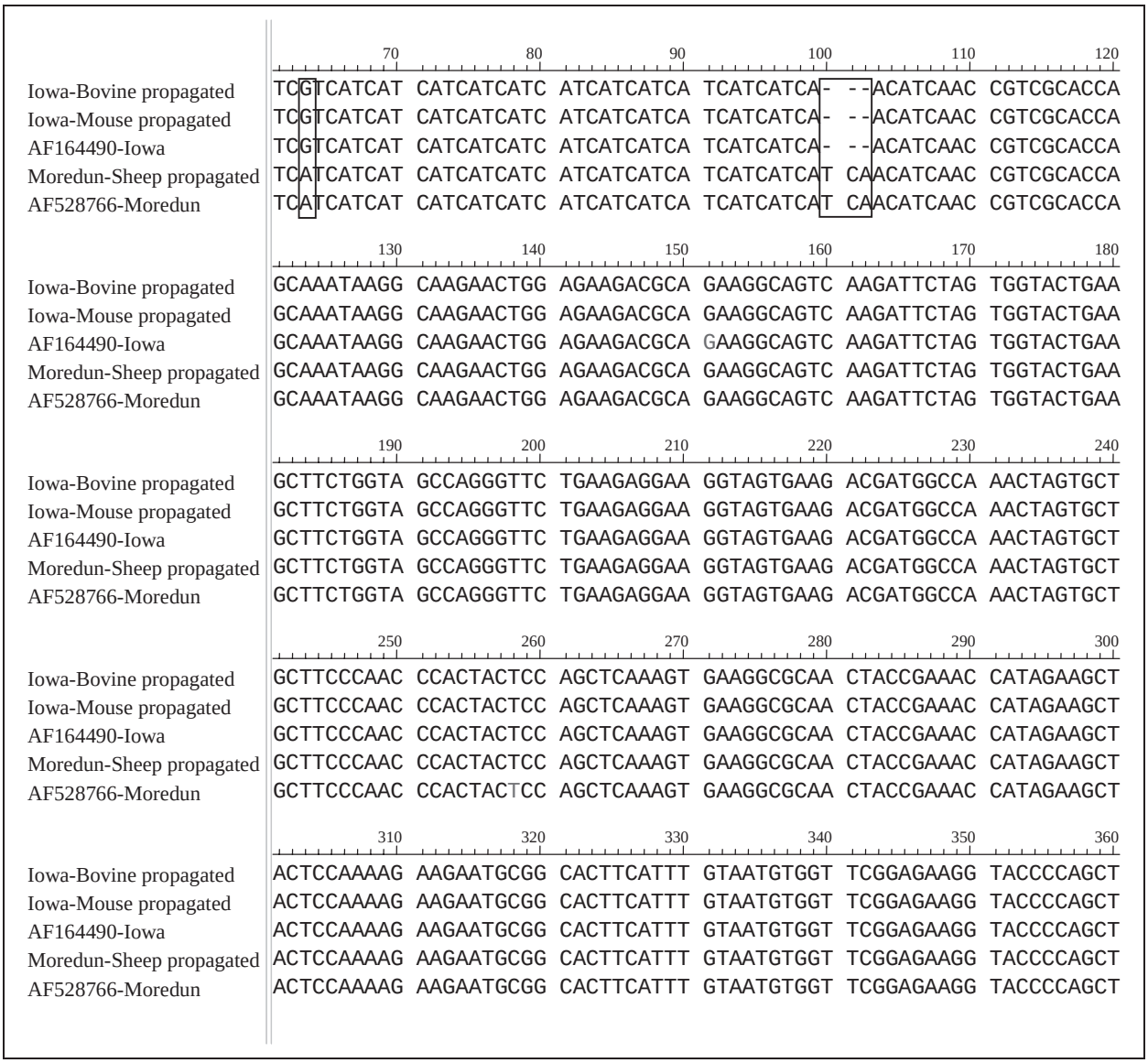


Figure 2.1 Alignment of a region of the *Cryptosporidium parvum* 60-kDa glycoprotein gene showing nucleotide differences (boxed) between the Iowa and Moredun isolates (positions 63 and 100–102)

slides were inoculated with 1,000 oocysts each. Cultures were incubated for 64–72 hours, stained using the IFA method, and the infectious foci counted. A minimum infectivity rate of 5% (50 infectious foci per monolayer) averaged across the six replicate monolayers with a CV of 50% was required for a lot of oocysts to be considered adequate for infectivity. For QA/QC purposes, oocysts were enumerated by IFA staining (Cellabs antibody) of 10 wells per USEPA Method 1623 (USEPA, 2005) with an acceptable CV of ≤16%.

The age of the oocysts post-shedding can have an effect on the infectivity of the oocysts. Oocysts that were 70 days old were approximately 6-fold less infectious than fresh oocysts (Rochelle et al. 2001). For the purposes of this study, most infectivity assays (>75%) were performed using oocysts that were less than 4 weeks post shedding. Unfortunately, this was not always possible.

Due to the fact that the oocysts are propagated in live animals, the availability of oocysts at specific ages is limited. However, the oldest oocysts used in this study were 6 weeks post shedding.

Pretreatment of Oocysts

Individual aliquots of oocysts were pretreated prior to infection of the HCT-8 monolayers. Oocysts were incubated in an acidified Hanks Balanced Salt Solution/1% Trypsin (AHBSS/T) for 1 hour at 37°C. Tubes were vortexed vigorously every 15 min. The oocysts were washed twice by adding fresh medium, centrifuging at 13,000 × g for 3 min, and then discarding the supernatant. The final pellet of oocysts was resuspended in fresh medium and used to inoculate the monolayer.

Oocysts were removed from 1,000 L water concentrates by immunomagnetic separation (IMS, Invitrogen). Before the oocysts were inoculated onto the cell monolayers, the magnetic beads were removed from the oocysts. All oocyst samples were incubated in AHBSS/T for 1 hour at 37°C with vigorous vortexing every 15 min. The sample was then placed on the magnet and the supernatant (containing the oocysts) was transferred to a fresh tube. An aliquot of fresh AHBSS/T was added to the magnetic beads and the sample was incubated at 37°C for an additional 5 min. The tube was placed on the magnet and the supernatant transferred to the tube containing the rest of the sample. The sample was then washed twice in fresh medium to remove all traces of trypsin before inoculating monolayers.

HCT-8 Cell Culture

Stock Cells

Monolayers of the human ileocecal adenocarcinoma cell line HCT-8 cell line (ATCC CCL-244; American Type Culture Collection, Rockville, MD) were grown and maintained at both laboratories. Cells were stored in liquid nitrogen. Stock cells were maintained in 150 cm^2 flasks and passaged twice a week in cell culture media containing RPMI-1640 with GlutaMAX (Invitrogen), 5% heat-inactivated fetal bovine serum (Hyclone), penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (0.25 µg/mL), and 20 mM HEPES buffer. Cells were passaged by adding 5 mL trypsin:EDTA (0.25% trypsin:0.02% EDTA in HBSS, Sigma) to the monolayer and incubating for 5 min at 37°C to release the cell monolayer from the flask, inactivating the trypsin by adding an equal volume of cell culture medium, centrifuging the cells at 160 × g for 5 min, and resuspending the cells in fresh cell culture medium. The cells were enumerated using a hemacytometer and 4×10^6 cells per flask were inoculated into a new 150 cm^2 flask containing 50 mL fresh medium. The cells were maintained in a humidified incubator at 37°C, 5% CO_2. Cells were not used beyond passage 30. Separate biological safety cabinets and incubators were used for uninfected stock cells and the infected monolayers.

Quality Assurance/Quality Control of Stock Cells

A strict QA/QC procedure was followed to ensure the health and integrity of the cells used for infectivity throughout the project. The complete cell culture medium was tested for sterility before use. All of the components were added to the base medium and then this prepared medium was tested for the growth of microbial contaminants by inoculating aliquots into Sabaroud-Dextrose agar, blood agar, brain heart infusion broth, and thioglycollate broth (PML

Microbiologicals, Wilsonville, OR). Approximately 5% of all prepared cell culture medium was used for QC purposes. If any batch of prepared cell culture medium tested positive for bacterial or fungal growth, it was discarded along with any cells that were grown in it.

Whenever a new batch of HCT-8 cells was thawed, the cell line was tested for the presence of contaminating mycoplasma. Some studies have shown that 64% percent of certain types of cell lines are contaminated with mycoplasma (Drexler et al. 2002). Although mycoplasma is so small that it usually cannot be seen under the microscope, it can adversely affect the function of the cell line. Antibiotics that are normally present in the complete media will not eradicate a mycoplasma contamination but it can slow its growth. When a new batch of HCT-8 cells was prepared, it was passaged twice in medium without antibiotics to allow for maximum confluence of the mycoplasma, if present. The cells were then tested for the presence of mycoplasma by Bionique Testing Laboratories (Saranac Lake, NY) which provides a testing service that stains the cells using a direct DNA fluorochrome staining technique. All cells used for this project were certified to be mycoplasma-free before being used for any infectivity assays.

QA/QC of Tissue Culture Facility

All culture of uninfected cells was performed in a dedicated area of the laboratory. Biological safety cabinets (BSC) were certified every 6–12 months by an accredited contract service to ensure operation within specifications. In addition, MWDSC conducted monthly QC checks on all BSCs by placing uncovered Sabaroud-Dextrose agar and blood agar plates in the hood for 15 min and then incubating the plates for five days and checking for growth. If a biological safety cabinet fails a QC check it is removed from service until repaired and recertified. No BSC failed during the course of this project.

Incubators were cleaned with an antimicrobial agent every two weeks. Biocidal ZF (Wak-Chemie Medical GmbH, Germany) was applied to the walls of the incubator according to the manufacturer's directions. The water tray in the incubators was cleaned and fresh water containing an antifungal agent (AquaClean, Wak-Chemie Medical GmbH, Germany) to reduce the possibility of fungal growth in the humidified incubator was added. Incubators were checked for contamination by placing uncovered Sabaroud-Dextrose agar and blood agar plates in the cleaned incubator for 15 min and then incubating the plates for five days and checking for growth.

Cell culture log sheets were maintained to record cell line information, passage number, date frozen, date thawed, type of media, size/type of flask, and lot numbers for FBS, trypsin, antibiotics, and all other media components. The logs provided a fully cross-referenced database that allows any media component or chemical used for any cell culture experiment to be traced to the original manufacturer, lot number, and date received.

Preparation of Cell Monolayers for Infectivity Assays

Although the methods all used HCT-8 cells, the three published *Cryptosporidium* infectivity assays differed in their assay formats, cell culture media formulations, and incubation periods. Ideally, the method comparison phase of the project would have combined each infection detection procedure with each set of medium formulations and incubation conditions. However, this was not practical within the constraints of the project budget and timeframe. Therefore, only some aspects of cell culture were standardized during the method comparison phase. The same HCT-8 maintenance medium was used for all assays, regardless of the eventual infection detection method

(Figure 2.2). However, following inoculation with oocysts, cells were grown in the medium specific to each published detection assay (Figure 2.2). This ensured that, as far as possible, the detection assays were applied to cells maintained under optimum conditions for each particular assay.

A stock flask of HCT-8 cells was split into two 150 cm^2 flasks of fresh maintenance medium. One flask, the assay flask seeded with 5×10^6 cells, was used to set-up the 96-well plates (PCR and RT-PCR detection assays) and 8-well chamber slides (Lab-Tek II, IFA detection assay) while the second became the new stock flask. The assay flask was incubated for 42–52 hours (80–100% confluence) and the monolayer was then lifted by trypsinization (see above for details). The cell suspension was seeded into 96-well plates and 8-well slides at a density of $9–10 \times 10^4$ cells/cm^2 surface area. After 42–52 hours incubation at 37°C, the maintenance medium was removed and monolayers were inoculated with oocysts suspended in the growth medium specific to each detection assay.

Detecting Infection by Immunofluorescence Microscopy

Monolayer Inoculation

Oocyst samples that were treated with AHBSS/T were inoculated onto HCT-8 monolayers (at least 80% confluent) in 8-well chamber slides. The maintenance medium was removed and a small volume (approx. 100 µL) of IFA growth medium (Figure 2.2) was added to each well to prevent the monolayers from drying out during the inoculation procedure. The oocyst sample was resuspended in the IFA growth medium and added to the well in a final volume of 500 µL. The inoculated chamber slides were then incubated at 37°C for 64–72 hours in a 5% CO_2 humidified incubator.

Staining Monolayers

For the purposes of this study the chamber slides were incubated for 64–72 hours after inoculating the monolayers. Although the number of infectious foci does not increase after 48 hours (Di Giovanni and LeChevallier 2005; Rochelle et al. 2001; Slifko et al. 1997), the number of stages present in the foci does increase, making enumeration of foci more reliable. The monolayers were then stained to allow visualization and enumeration of infectious foci. The medium was removed from the wells and the monolayers immediately fixed with methanol for 10 min. At the MWDSC laboratory, methanol was removed from the wells and the chambers removed from the slides following the manufacturer's instructions. Monolayers were then incubated in the blocking buffer (PBS, 2% goat serum, 0.002% Tween-20) for 30 min at room temperature. After removal of the blocking buffer, the rat anti-*Cryptosporidium* sporozoite antibody (Waterborne, Catalogue number #A600, unlabeled) diluted 1:500 in 1× PBS was added to the monolayer. The slides were then incubated in a humidified chamber for 45 min at room temperature. After four washes in 1× PBS, secondary goat anti-rat IgG FITC labeled antibody (Sigma F6258) diluted in 1× PBS (1:150 dilution) was added to the monolayer and slides incubated for an additional 45 min. The antibody was removed with four washes in 1× PBS and the slides allowed to dry. Coverslips were applied over mounting medium (Waterborne).

At the AgriLife El Paso laboratory the chambers were left intact on the slide after the monolayers were fixed with methanol, the blocking buffer and then the stain was added to the individual chambers and the slides incubated. The monolayers with the chambers still on the slides were analyzed using an inverted-phase contrast microscope equipped with epifluorescence.

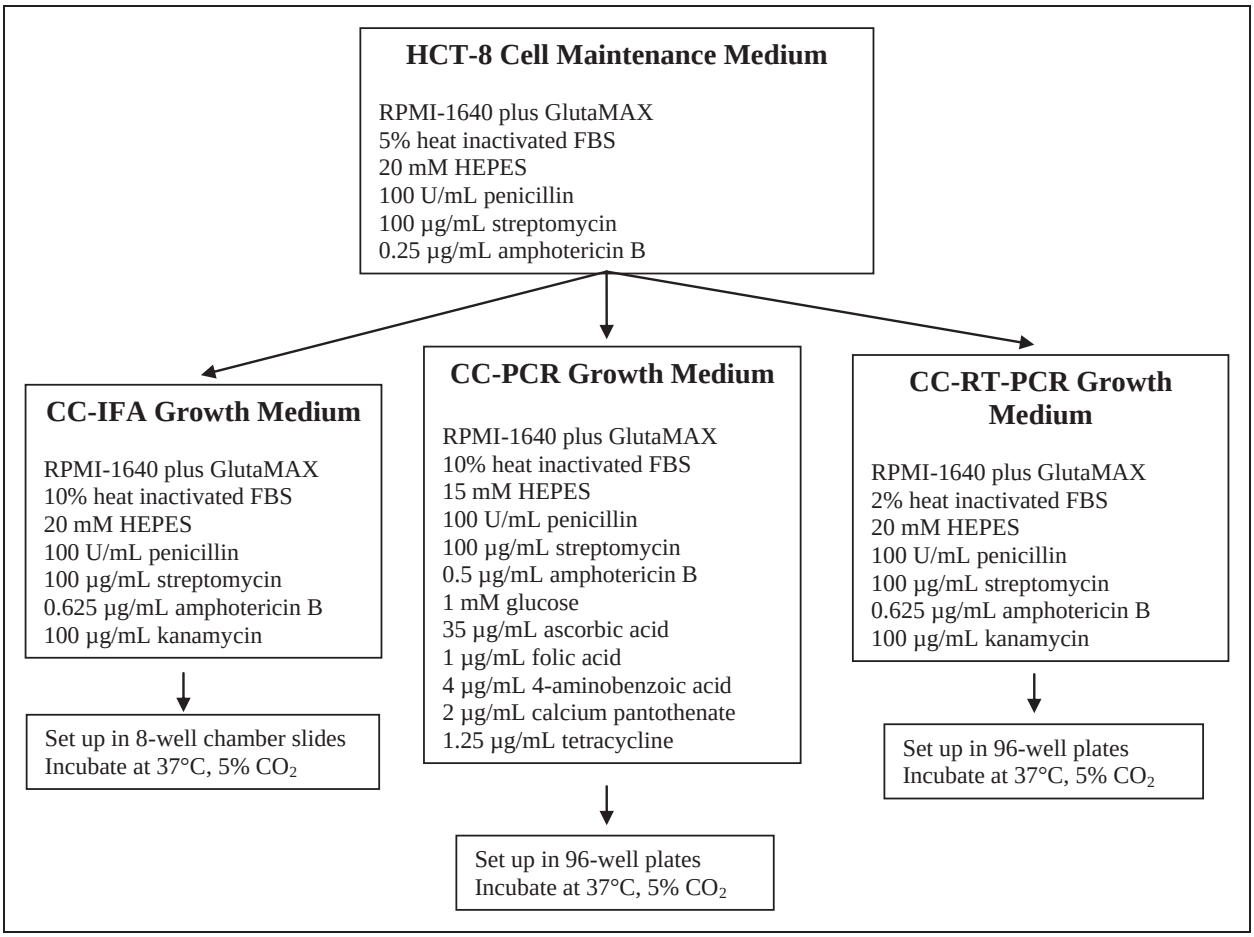


Figure 2.2 Cell culture media. HCT-8 cells were maintained in the maintenance medium until the monolayers were infected. At the time of infection, the growth medium for the specific infectivity assay was added to the monolayers.

Counting Infectious Foci

There is inconsistency in published work on the definition used to describe cell culture infection. Since infections were quantified for QA/QC purposes and maximum sensitivity of infection detection avoiding false-positives was necessary for phase 2 of the project, a uniform measure of infection was necessary. Therefore, the investigators agreed upon definitions based on microscopic measurements of infections detected by IFA and colorimetric in-situ hybridization (CISH).

Infectious foci have previously been defined based on CISH as a focus of life stages in closer proximity to each other than to other foci, a non-quantitative subjective definition (Rochelle et al. 2001). Foci diameters ranged from 12 to 144 µm, generally depending on the number of individual stages in the focus. For the current project, intracellular developmental stages and foci of stages were measured following detection of infection by IFA and CISH. There was no significant difference in the size of foci between IFA and CISH (P=0.44, 95% CI, n=38) or in the distance between stages within foci (P=0.062, 95% CI). Therefore, the following measurements are based on combined IFA and CISH data.

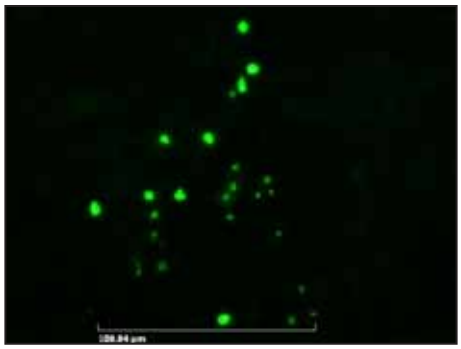


Figure 2.3 *Cryptosporidium parvum* infectious focus on HCT-8 monolayer detected by immunofluorescence microscopy

The size of infectious foci was measured as the largest diameter × the perpendicular diameter. Foci generated by the *C. parvum* Iowa isolate were 97.4 ± 36.3 µm × 62 ± 27.7 µm (mean ± standard deviation, n=79). The average distance between developmental stages within a focus was 7.8 ± 7.1 µm (n=117). The largest focus that could readily be considered a single focus, rather than two or more merged foci, was 175 µm in diameter.

For the purposes of this project, when IFA was used to detect infected monolayers, infection was defined as a monolayer that contained at least one focus of life stages. A focus of stages was defined as at least three life stages within an area ≤ 175 µm in diameter (Figure 2.3). The separation between the perimeters of distinct foci should be at least 23.4 µm (average distance between stages × 3). An individual life stage was defined as an intracellular life cycle stage × 3). An individual life stage was defined as an intracellular life cycle stage ≥1 µm and ≤10 µm in diameter, with the correct color and intensity of fluorescence, and not an obvious fluorescent artifact. Based on this definition, an inoculated monolayer that contained only one or two green fluorescing objects of the correct size and morphology was considered negative for infection.

Detecting Infection by Polymerase Chain Reaction

Inoculating Monolayers

Oocyst samples that had been treated with the AHBSS/T were inoculated onto HCT-8 monolayers (at least 80% confluent) in 96-well tissue culture plates. The medium the cells were maintained in was removed and fresh growth medium was added to each well according to the published PCR method (Figure 2.2). The oocyst sample was resuspended in the same growth medium and added to the well for a final volume of 100 µL. Plates were then incubated at 37°C for 64 to 72 hours in a 5% CO_2 humidified incubator.

Extracting DNA From Monolayers

Cell culture medium was removed from the wells and monolayers washed five times with 1× PBS. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA; catalogue number 51306). Mock infection inocula were not removed prior to the addition of DNA extraction reagents. DNA was eluted off the column by adding 50 µL 0.01× TE buffer, pH 8 preheated to 70°C and incubating the column at 70°C for 5 min.

Table 2.3
PCR amplification conditions

Reagent	Quantitative PCR	Conventional PCR
Buffer	1× TaqMan A	1× AmpliTaq Gold
$MgCl_2$	3.0 mM	2.5 mM
dNTPs	200 µM dGTP, dCTP, dATP 800 µM dUTP	200 µM dGTP, dCTP, dATP 800 µM dUTP
Primers	200 µM CPHSPT2-F, CPHSPT2-R	200 µM cphsp 2423F, cphsp 2764R
Probes	200 µM CPHSP2P2	NA
BSA	1.5 mg/mL	1.5 mg/mL
Polymerase	0.05 U/µL AmpliTaq Gold	0.05 U/µL AmpliTaq Gold
Uracil DNA glycosylase	0.01U/µL	0.01 U/µL

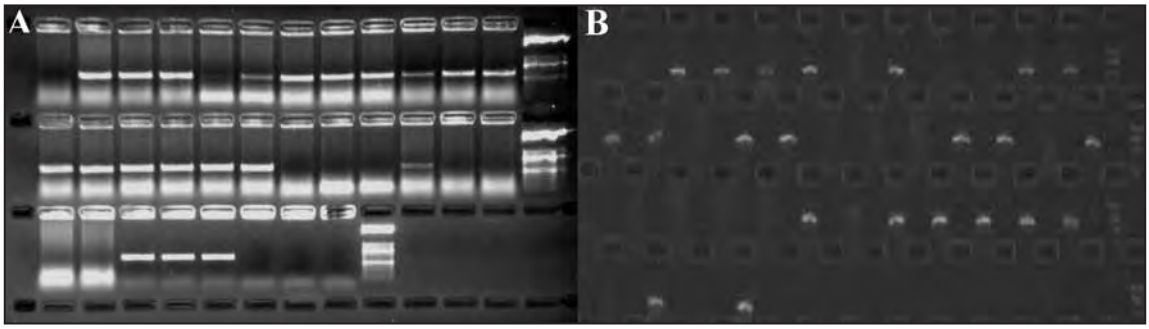


Figure 2.4 Examples of infection detection by conventional PCR (A) and RT-PCR (B)

Amplifying Cryptosporidium*-Specific DNA by PCR*

DNA was amplified by either conventional PCR at MWDSC or quantitative PCR at AgriLife El Paso. The basic amplifying reagents and conditions were the same at both laboratories (Table 2.3) except that the quantitative PCR included the TaqMan probe. The entire 50 µL of DNA was used in a 100 µL amplification reaction.

Gel Electrophoresis of Amplicons

The high concentration of BSA in amplification reactions caused a white precipitate to form in the reaction mixture. Therefore, PCR samples were centrifuged prior to loading the samples on a gel to prevent BSA from being transferred onto the gel and interfering with amplicon migration. An aliquot of each sample (10% of the total volume at AgriLife El Paso and 20% at MWDSC) was mixed with Orange G loading buffer and loaded onto a 1.5% agarose gel in 1× TBE. The gels were run at 85V for approximately 1 hour, then stained with ethidium bromide (0.5 µg/mL) for 30 min and destained for 30 min. The bands were visualized with a UV transilluminator and photographed (Figure 2.4A). PCR amplification reaction products were detected by real-time TaqMan PCR and gel electrophoresis at AgriLife El Paso and by electrophoresis only at MWDSC.

Detecting Infection by RT-PCR

Inoculating Monolayers

Oocyst samples that had been treated with the AHBSS/T were inoculated onto HCT-8 monolayers (at least 80% confluent) in 96-well tissue culture plates. The maintenance medium the cells were maintained in was removed and new growth medium was added to each well according to the RT-PCR method (Figure 2.2). Oocysts were resuspended in the same growth medium and added to the well for a final volume of 100 µL. Plates were then incubated at 37°C for 64–72 hours in a 5% CO_2 humidified incubator.

Extracting RNA From Monolayers

Cell culture medium was removed from monolayers, which were then washed twice with 1× PBS. Cells were then lysed and RNA extracted using a Qiagen RNeasy 96 RNA extraction kit (Qiagen, Valencia, CA, Catalogue number 74181). Residual DNA that may have carried over in the RNA extraction was removed by treating the filters twice with 80 µL DNAse 1 (1,800 KU/mL) for 20 min at 37°C. The RNA was eluted in 80 µL of RNase-free water.

Amplifying Cryptosporidium-Specific RNA by RT-PCR

RNA was reverse transcribed to cDNA using Murine Leukemia Virus reverse transcriptase (MuLV-RT; 2.5U/µL), RNase inhibitor (1 U/µL), oligo $d(T)_{16}$ primers (2.5 µM), and 10 µL of RNA in a 20 µL reaction. The entire 20 µL RT reaction was used as the template for the amplification reaction. The amplification reaction consisted of $MgCl_2$ (1.5 mM); dATP, dCTP, dGTP, and dUTP (200 µM each); the forward and reverse primers (0.25 µM each; Table 2.5); uracil DNA glycosylase (0.01 U/µL); and Platinum Taq Polymerase (0.025 U/µL). The cDNA was amplified using conventional PCR at both laboratories.

If DNA was inadvertently extracted along with the RNA in the extraction procedure, the DNA could possibly carry through to the PCR and give a false positive result. Therefore, a separate RT reaction for each RNA sample was set up without the MuLV-RT. The product of this reaction was then amplified in the PCR reaction. If carryover DNA was present in the RNA sample, the PCR reaction would amplify this DNA. Therefore, it would imply that a positive result was from the presence of contaminating DNA since RNA was not reverse transcribed into cDNA (no MuLV-RT enzyme in the reaction). Reactions that were set up without MuLV-RT and were positive, were interpreted as false-positive samples.

Gel Electrophoresis of Amplicons

An aliquot of each sample (10% of the total volume at AgriLife El Paso and 20% at MWDSC) was mixed with Orange G loading buffer and loaded onto a 1.5% agarose gel in 1× TBE. The gel was run at 85V for about 1 hour, then stained with an ethidium bromide solution for 30 min and destained for 30 min. The bands were visualized with a UV transilluminator and photographed (Figure 2.4B). The molecular weight of the amplicon was confirmed by comparing to molecular size standards.

Method	Sequence (5'-3')	Size	Reference
RT-PCR	F-AAATGGTGAGCAATCCTCTG R-CTTGCTGCTCTTACCAGTAC	361 bp	Rochelle et al. 1997 Rochelle et al. 2002
PCR	F-TCCTCTGCCGTACAGGATCTCTTA R-TGCTGCTCTTACCAGTACTCTTATCA	346 bp	Di Giovanni and Aboytes 2003

Figure 2.5 Infection detection primers for RT-PCR and PCR

Primers for PCR and RT-PCR

The protocols for both the PCR and RT-PCR detection methods use primers that target the *Cryptosporidium* hsp70 gene (Figure 2.5). The amplicon produced by the PCR primers overlaps the amplicon produced by the RT-PCR primers. Although these primers were designed to target the *C. parvum* hsp 70 gene, Figures 2.6 and 2.7 show that they can also be used to detect *C. hominis.*

Genotyping Positive Samples

The cDNA and DNA resulting from the RT-PCR and PCR methods, respectively, is a good source of DNA that can be used for genotyping of any positive samples. For the IFA method, which does not rely on DNA amplification, infected HCT-8 monolayers were fixed and stained with the anti-sporozoite antibody (FITC-labeled). Infectious foci on the monolayer were visualized using epifluorescence microscopy and lysis buffer was applied to the area of the monolayer containing the infectious focus. The affected area of the monolayer was then scraped off the slide using a sterile micropipette tip. The lysate was then transferred to a sterile microcentrifuge tube containing additional lysis buffer. DNA extraction was performed using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, Catalogue number CS11200).

Controls in Infectivity Assays

Mock Infections

To properly assess the method, every assay had controls for false positives and false negatives. Mock infections entailed inoculating monolayers with 25 flow cytometry sorted oocysts per well in 5 wells per assay, immediately before processing the sample. For the RT-PCR and PCR method, the cell culture medium was removed, the monolayer washed with 1× PBS, and the mock infection controls inoculated onto the monolayer. The lysis buffer was then immediately added to the monolayer to lyse the cells. For the IFA method, the mock infection oocysts were inoculated onto the monolayer after the cell culture medium was removed from the cells but before the monolayer was fixed with methanol. The mock infection wells were then processed along with the other

Species	Isolate	Forward primer (5'-3')* TCCTCTGCCGTACAGGATCTCTTA	Reverse primer (5'-3')* TGCTGCTCTTACCAGTACTCTTATCA
C. parvum	Iowa Moredun KSU-1 Ferret Human Mouse	....T.....	....
C. hominis	TU502 A29 A5	....	.....G..T..... .....G..T..... .....G..T.....
C. meleagridis	Quail Human Turkey	.....T..... .....T..... .....T.....	....
C. wrairi	Guinea pig	.....T.....	.....
Cryptosporidium sp.	Opossum	..T.....T..T..A..C..T...	.....T.....
Cryptosporidium sp.	Kangaroo	..T.....T..T..A..C..T...	.....T.....
C. serpentis	Tree boa	..A..A.TA.....A...T.A...	.....T.....G.....
C. suis	Pig	..T.....T..T..A.....G	....A.....T.....T
C. baileyi	Quail	..A..A..T.....T.A...	.A.....
C. canis	Coyote	.....C.....C.....G	.....G..G.....G...
C. felis	Cat	..T.....G..C..A..C..A..G	....A.....C.....G.....G...
C. andersoni	Cow	..A..A.TA..G..A...T.A..G	.....G.....G...
C. muris	Mouse	..A..A.TA..G..A...T.A..G	.....G.....G...

*Primer sequences were published by Di Giovanni and Aboytes 2003.

Figure 2.6 PCR primer specificity. Dots indicate identity.

samples. Since these oocysts did not have time to initiate an infection, detection signal produced by any of the methods would be a false-positive generated by oocysts on the monolayer but not active infections. This simulated intact but non-infectious oocysts remaining on the monolayers. In actual samples, any intact but non-infectious oocysts would probably be removed either when the cell culture medium was removed or during the PBS washing steps since they would not have attached to the cell monolayer. The addition of mock infection oocysts after these steps but before processing represents a worst case scenario of non-infectious oocysts attaching to the cell monolayer without initiating an infection.

Inactivated Controls

It was important that the assay selected for the survey of infectious oocysts in finished drinking water not give a false positive result when non-infectious oocysts were present. Oocysts were inactivated by a variety of methods, flow cytometry enumerated and sorted into individual

Species	Isolate	Forward primer (5'-3')* AAATGGTGAGCAATCCTCTG	Reverse primer (5'-3')* CTTGCTGCTCTTACCAGTAC
C. parvum	Bovine (Iowa) Moredun KSU-1 Ferret Human Mouse	....	....
C. hominis	Human (TU502) Human (A29) Human (A5)	....	.....G. .....G. .....G.
C. meleagridis	Quail Human Turkey	....	....
C. wrairi	Guinea pig	.....	.....
Cryptosporidium sp.	Opossum	.....T....	.....
Cryptosporidium sp.	Kangaroo	.....T....	.....
C. serpentis	Tree boa	.....A.....A..A.	.....T.....G....
C. suis	Pig	...C.....A..G..T....	......A.....
C. baileyi	Quail	......A..A.....A..A.	T..A.....
C. canis	Coyote	G..C.....C.	.....G..G.
C. felis	Cat	G..C.....G..T....	......A.....C.....G.
C. andersoni	Cow	.....A..G..A..A.	.....G....
C. muris	Mouse	.....A..G..A..A.	.....G....

*Primer sequences were published by Rochelle et al. 1997.

Figure 2.7 RT-PCR primer specificity. Dots indicate identity.

tubes by WSLH, and inoculated onto cell monolayers to determine if they were capable of giving a false positive result for infectivity.

Gamma-irradiated (0.5 kGy) EasySeed oocysts were originally supplied by BTF (Australia). The later studies were performed with oocysts that were irradiated (0.5 kGy) by Food Technology Services, Inc. (Florida). An initial infectivity trial at MWDSC using the RT-PCR method to detect infections demonstrated no infections in 10 replicate monolayers inoculated with 100 gamma-irradiated oocysts each. This radiation dose is recommended by the U.S. Food and Drug Administration for inactivation of protozoa on food.

Oocysts were irradiated with approximately 60 mJ/cm^2 of low-pressure UV using previously described equipment and procedures (Rochelle et al. 2004). UV-irradiated oocysts were then enumerated and sorted into individual tubes containing 10 and 100 oocysts by flow cytometry at WSLH. A UV dose of 40 mJ/cm^2 irreversibly inactivates at least 99.99% of an oocyst population (Rochelle et al. 2004).

Oocysts were heat inactivated by incubating at 70°C for 30 min followed by cooling to room temperature. They were enumerated and sorted by flow cytometry at WSLH into individual tubes before being used for infectivity assays.

Viable oocysts were frozen at –80°C for 18 h and then thawed at 95°C for 2 min. These oocysts were then enumerated and sorted by flow cytometry at WSLH into individual tubes before being used for infectivity assays.

Trip Control Oocysts

Oocysts used for the infectivity assay were tested by both laboratories (MWDSC and AgriLife El Paso) for initial infectivity as previously described in the QA/QC section. Once the batch of oocysts demonstrated a minimum infectivity rate of 5%, they were enumerated and sorted into individual tubes by flow cytometry at WSLH for the method comparison infectivity assays. Two additional tubes of oocysts accompanied the flow cytometry sorted oocysts as trip controls. These oocysts were not processed in any way at WSLH but were returned to the laboratories (one tube to each laboratory) with the sorted oocysts. The trip control oocysts were then used in infectivity assays to ensure that the oocyst infectivity had not been adversely affected during shipping.

Definition of Infection

There are different ways of expressing infectivity. For the purpose of this project, infectivity was expressed as either proportional infectivity (Equation 2.1) or percent infectivity (Equation 2.2). Percent infectivity can be based on either the total number of oocysts inoculated onto a monolayer or the number of infectious oocysts (as previously determined by assessing the infectivity of each lot of oocysts). For the RT-PCR and PCR methods, proportional infectivity was used to indicate the number of wells with a positive result for infectivity per the total number of wells inoculated. Infectivity for the IFA method could be expressed as either proportional or percent infectivity since the IFA method allows for quantification of individual infectious focus on the monolayer. The number of infectious oocysts was calculated for each lot of oocysts from the initial QA/QC infection.

$$\text{Proportional infectivity}\,(\%) = \left(\frac{\text{Number of wells that develop infection}}{\text{Number of wells inoculated at each challenge dose}}\right) \times 100 \quad (2.1)$$

$$\text{Percent infectivity}\,(\%) = \left(\frac{\text{Number of infection foci}}{\text{Number of oocysts inoculated onto monolayer}}\right) \times 100 \quad (2.2)$$

Proportional infectivity should decrease as the oocyst challenge dose is reduced. Conversely, percent infectivity based on the number of infectious foci should remain relatively constant, irrespective of the oocyst challenge dose. Examples of infectivity quantification using these different methods are provided in Table 2.4.

Table 2.4
Infections detected by immunofluorescence assay*

Flow-sorted oocysts	No. replicate wells	Measures of infectivity							
		MWDSC				AgriLife El Paso			
		Positive wells	%†	Average foci per monolayer	%‡	Positive wells	%†	Average foci per monolayer	%‡
500	5	5	100	83	17	5	100	59	12
25	10	10	100	4.7	19	9	90	2.7	11
10	10	8	80	2	20	3	30	0.5	5
5	10	7	70	0.8	16	5	50	1.2	24
1	10	3	30	0.33	33	0	0	0	0
Mean					21				13
s.d.					6.9				7.9

*Mouse propagated oocysts (Iowa isolate) were 21 days post-shedding at the time of monolayer inoculation. Infections prior to flow cytometry demonstrated 16.1% and 17.4% infectivity at MWDSC and AgriLife El Paso, respectively, based on the number of infection foci per inoculum oocyst.
†Proportional infectivity (%): Number of positive wells per number of wells inoculated ×100.
‡Percent infectivity (%): Average number of foci per monolayer per number of oocysts inoculated.

METHOD EVALUATION RESULTS

Method Optimization and Standardization

Oocyst Isolates and Sources

There appears to have been a reduction in the overall quality, consistency, and infectivity of *Cryptosporidium* oocysts that are available for research. Therefore, oocysts from a variety of sources were evaluated using the cell culture/RT-PCR method at MWDSC. Bovine-propagated and mouse-propagated oocysts (*C. parvum* Iowa isolate) were obtained from Waterborne, Inc. (New Orleans, LA). Bovine propagated *C. parvum* Iowa oocysts were also obtained from the University of Arizona (Tucson, AZ) and Bunch Grass Farms (Deary, ID; previously called Pleasant Hill Farms) and the *C. parvum* Moredun isolate, propagated in sheep, was provided by Steve Wright (Moredun Institute, Penicuik, Scotland).

All oocysts were subjected to rigorous quality control evaluation prior to use for infectivity experiments. This included microscopic observation of wet mounts, Gram-stained samples, and FITC-antibody stained samples and inoculation of nutrient broth, Sabaroud-dextrose plates, m-endo plates, and RPMI-1640 cell culture medium containing antibiotics and 2% FBS. Seventy-five percent of oocyst lots from the University of Arizona during the 12 months prior to this study were contaminated with bacteria and/or yeast, and four (50%) were contaminated with *Escherichia coli*, including three (37.5%) that contained antibiotic-resistant *E. coli* able to grow in cell culture medium. When present, the concentration of *E. coli* was typically 0.01–5 colony forming units (CFU) per 10^3 oocysts. Such contamination renders these oocyst preparations unsuitable for cell culture applications since the contaminating bacteria usually overgrow and kill the cells. During the four year period prior to 2004, 11 of 46 (23.9%) oocyst lots from the University of Arizona were contaminated with *E. coli* but only three of these (6.5%) were able to grow in cell culture medium containing antibiotics. While most of the oocyst lots received from Waterborne were

contaminated with bacteria, *E. coli* contamination had not been detected (N = 11). The single preparation of oocysts received from Bunch Grass Farms was very heavily contaminated with bacteria and yeasts that overgrew the cell culture medium. Although most bacterial and yeast contaminants can be eliminated by treatment of the oocysts with 0.525% (w/v) sodium hypochlorite prior to inoculation of cell monolayers, the infectivity methods used in this project did not include oocyst treatment with bleach. Consequently, minimal contamination of oocyst preparations was required for these methods although they included antibiotics in the cell culture medium.

The average historical ID_{50} for untreated Iowa oocysts from the University of Arizona when analyzed by the HCT-8 cell culture/RT-PCR method was 78 based on 31 dose response curves generated over a five year period (Rochelle et al. 2002, 2004). More recent ID_{50} values, obtained using the same method, were 224 and 3,119 oocysts (MWDSC, unpublished data), indicating considerably decreased infectivity. Reduced infectivity of oocysts supplied by the University of Arizona has also been noted at AgriLife El Paso and OCU. Recent shipments of mouse-propagated oocysts (Iowa isolate) from Waterborne had average ID_{50} values of 27–40. Therefore, mouse-propagated oocysts of the Iowa isolate supplied by Waterborne, Inc. were used for most of the method evaluations and comparisons.

Detecting Infection With Various Isolates of Cryptosporidium

The Iowa and Moredun isolates were compared in an initial trial of all three infectivity methods at MWDSC. The Iowa isolate oocysts were 28 days old (post-shedding) at the time of cell culture inoculation and Moredun oocysts were 70 days old. The standard QA infectivity assessment adopted for this project (IFA) was conducted on 3-day old oocysts of the Iowa isolate and demonstrated 86.8 ± 31.7 (mean ± standard deviation, N = 6 wells) infectious foci per 1,000 inoculum oocysts (8.7% infection rate) meeting our minimum acceptable infection rate of 5% for QA purposes. Oocysts were sorted by flow cytometry (Wisconsin State Laboratory of Hygiene) so that challenge doses of 1, 5, 10, and 25 oocysts could be inoculated onto each monolayer (10 monolayers per challenge dose). In accordance with the individual methods, oocysts were incubated in acidified trypsin prior to monolayer inoculation for the RT-PCR and PCR-based detection methods. Although in most instances oocysts were incubated in acidified trypsin prior to inoculation of the monolayer, in this case oocysts used for the IFA detection method were treated with bleach.

The results of this comparison are presented in Table 2.5. Although there was considerable variability within the results and the lowest oocyst dose that consistently generated detectable infection across all three methods and both isolates, the results demonstrated that each method was capable of detecting infection. In this preliminary trial, the only method that generated a positive result with a single flow cytometry sorted oocyst was RT-PCR with the Moredun isolate. Based on just the RT-PCR results, this lot of Moredun oocysts had an ID_{50} of 13. Our previous studies with this isolate have demonstrated an ID_{50} of 9–25 (95% confidence level) in CD-1 mice and 18–38 in HCT-8 cell culture (Rochelle et al. 2002). A second comparison of isolates generated an ID_{50} of 19 for the Moredun isolate and 58 for Iowa (Figure 2.8).

Most cases of cryptosporidiosis in humans are caused by either *C. parvum* or *C. hominis* oocysts. The three cell culture infectivity detection methods were tested to determine their efficiency in detecting the presence of *C. hominis* oocysts. Oocysts of *C. hominis* (isolate Tu728) were propagated in gnotobiotic piglets (Giovanni Widmer, Tufts University) and used for infections between 7 and 21 days post shedding. Oocysts were enumerated by flow cytometry at WSLH and sorted into individual tubes containing 100, 50, 25, 10, and 5 oocysts per tube (10 tubes per

Table 2.5
Preliminary comparison of three infectivity methods at MWDSC

Oocysts	Dose per well*	Infectivity detected by: IFA	PCR	RT-PCR
C. parvum Iowa isolate	25	20%	30%	20%
	10	0	20%	0
	5	0	0	0
	1	0	0	0
C. parvum Moredun isolate	25	0	20%	80%
	10	10%	10%	20%
	5	0	20%	30%
	1	0	0	10%
Mock infection	25	0	0	0
Unseeded	0	0	0	0

*Each challenge dose was inoculated into 10 wells of cell culture.

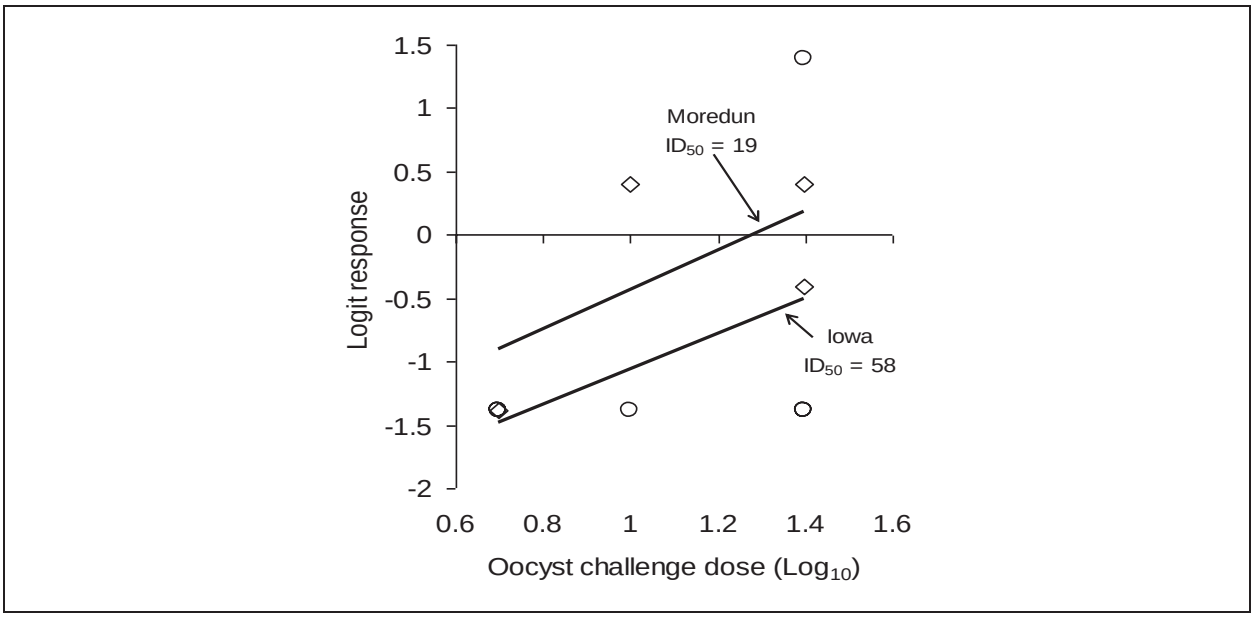


Figure 2.8 Dose response curves for the *C. parvum* Iowa (○) and *C. parvum* Moredun (◊) isolates. Infections were detected by RT-PCR.

dose). The oocysts were subjected to AHBSS/T pretreatment and inoculated onto cell monolayers. All of the infectivity detection methods detected *C. hominis* infections although only the IFA and PCR methods detected infection with 5 and 25 oocysts (Table 2.6). Multiple developmental stages were observed in the *C. hominis* infectious foci and the anti-sporozoite antibody used for the IFA detection method is therefore capable of detecting *C. hominis* as well as *C. parvum* developmental stages.

Acidification of Oocysts Prior to Monolayer Inoculation

The usual pretreatment for oocysts that are to be used for infection and detection by immunofluorescence is incubation in 0.525% (w/v) sodium hypochlorite to simultaneously activate

Table 2.6
Comparison of methods for measuring *C. hominis* infectivity in cell culture

		Proportional infectivity (%)		
Oocysts/well	N*	IFA	PCR	RT-PCR
100	10	60	90	100
50	10	50	40	30
25	10	20	20	<10
5	10	10	10	<10
Mean % infectivity (total)†		1.5%	NA‡	NA
Mean % infectivity (infectious)§		125%	NA	NA

*Number of replicate monolayers inoculated with indicated oocyst dose.
†Based on the number of infectious foci per total oocysts inoculated (QC infectivity = 1.2%).
‡Based on the number of infectious foci per infectious oocysts inoculated.
§Not applicable because the PCR and RT-PCR methods did not allow enumeration of infectious foci.

Table 2.7
Comparison of oocyst pretreatment methods

	% infectivity*	
	MWDSC	AgriLife El Paso
0.2 M HCl only	ND†	2.7 ± 0.9
0.2 M HCl/0.525% NaOCl	0.4 ± 0.1	1.4 ± 1.5
0.2 M HCl/0.0525% NaOCl	11.3 ± 2.7	ND
AHBSS/trypsin‡	13.7 ± 2.3	10.7 ± 0.7

*Calculated as the number of infection foci per inoculum oocyst.
†Not done.
‡Acidified Hanks Balanced Salt Solution/1% trypsin.

oocysts and inactivate any contaminating bacteria or yeast in the oocyst preparation. Since the survey phase of the project involvzzed acid disassociation of IMS-purified oocysts prior to monolayer inoculation, this pretreatment procedure was modified to mimic the acidification step. Oocysts were incubated in 0.2 M HCl at room temperature for 20 min followed by neutralization in 1 M NaOH, and then incubated in 0.525% (w/v) sodium hypochlorite at room temperature for 8 min and washed in PBS. However, this procedure generated inconsistent and generally poor rates of infection compared to the acidified Hanks Balanced Salt Solution (AHBSS) and trypsin oocyst pretreatment conditions that are used for the PCR and RT-PCR based detection methods (Table 2.7). Consequently, although it differs from the published procedures for detection of infection by IFA, AHBSS/T was used for all oocyst pretreatments, regardless of the final detection method.

Effects of Sodium Hexametaphospate on Oocyst Infectivity

Since addition of sodium hexametaphosphate (HMP) is necessary for efficient recovery of oocysts from large volume samples (see Chapter 3), the effect of this compound on the infectivity of oocysts was evaluated in three experiments. Two lots of viable mouse-propagated oocysts (Iowa) were obtained from Waterborne. These oocyst preparations contained a relatively low density of gram-positive and gram-negative bacterial contaminants that grew in non-selective QC media but not in cell culture medium containing 2% FBS. An average of 31% was DAPI-positive.

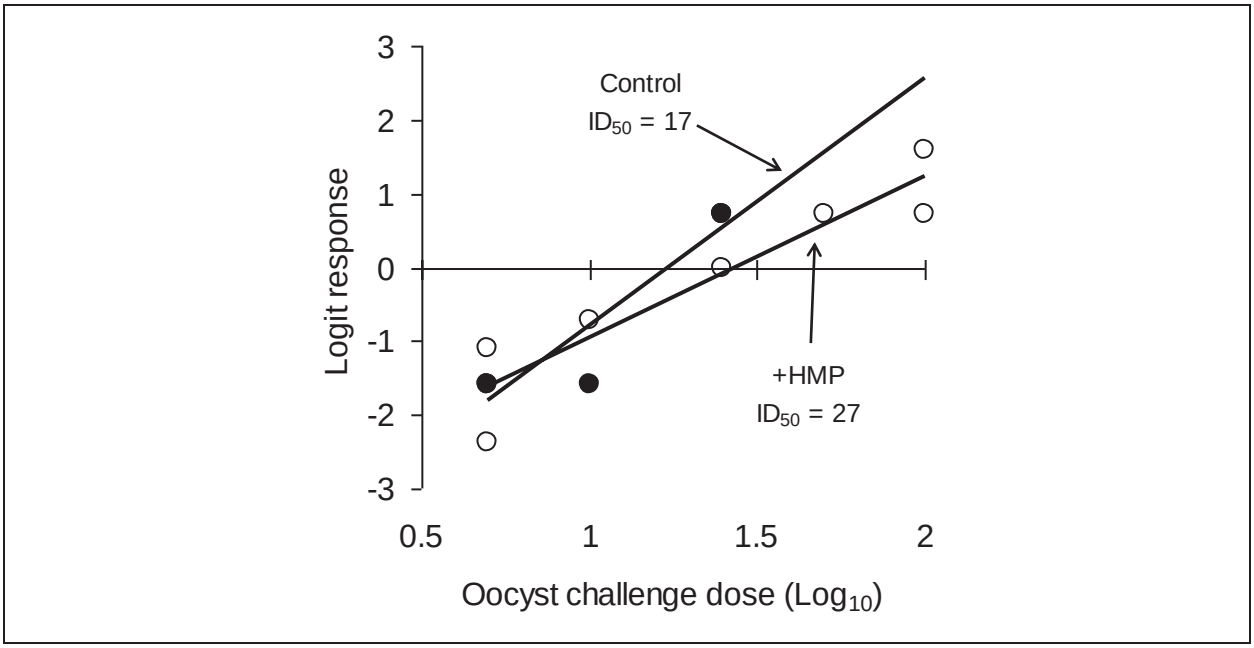


Figure 2.9 Dose response curve of sodium hexametaphosphate (HMP)-treated *C. parvum* Iowa oocysts (○) compared to untreated controls (●). Infection was detected and quantified by RT-PCR.

Table 2.8
Effect of sodium hexametaphosphate on oocyst infectivity

Oocyst dose	Proportional infectivity (%)*	
	Control	+HMP
25	25	50
50	75	75
100	100	100

*Infections enumerated by immunofluorescence assay.

Oocysts were incubated in 5% HMP for 10 min at room temperature. Following removal of HMP, oocysts were incubated for 1 h at 37°C in acidified Hanks balanced salt solution (AHBSS) containing 1% trypsin with mixing by vortexing at 15 min intervals. After diluting the AHBSS/1% trypsin by addition of cell culture medium, oocysts were diluted and inoculated onto HCT-8 monolayers (5–100 oocysts per monolayer). Each oocyst challenge dose was inoculated onto duplicate sets of six monolayers and infection was detected by RT-PCR targeting *hsp70* gene transcripts (Rochelle et al. 1997, 2002). Infectivity was expressed as a logistic transformation of percent infectivity. There was no significant difference between the dose response curves of HMP-treated and control oocysts (Figure 2.9).

Oocysts were also inoculated onto chamber slides (4 slides per oocyst dose) and infections detected by IFA. Again, there was no difference between treated and untreated oocysts (Table 2.8). The average incidence of infectious foci per monolayer was 2.5% for controls compared to 3.3% for HMP treated oocysts (foci per inoculum oocyst, averaged across three oocyst doses: 25, 50, and 100 oocysts per monolayer). The experiments demonstrated that HMP had no effect on oocyst infectivity.

Standardization of Methods

At the beginning of Phase 1 of the project, a technology transfer workshop was held at the MWDSC laboratory. The purpose of this workshop was to develop standardized procedures for the two participating laboratories to minimize operator and procedural differences as sources of variability in experimental results. The workshop covered cell culture and maintenance techniques, oocysts inoculation procedures, nucleic acid extraction methods, IFA staining of infected monolayers, RT-PCR and PCR, gel electrophoresis, and epifluorescence microscopy. Also, the differences in standard operating procedures between each of the three method developers, and sources of reagents, supplies, and oocysts were reviewed.

It was important for this project that the assays were optimized for the maximum level of detection of infectious oocysts. The infectivity of different lots of oocysts can vary widely depending on various factors such as source, age post-shedding, and storage temperatures. A detailed oocyst QA/QC policy was developed during the workshop to determine the rate of infectivity of each lot of oocysts before the lot would be used in any cell culture infectivity assays (see Materials and Methods Section).

A list of media components for the three different cell culture infectivity and detection methods along with the suppliers was compiled during the technology transfer workshop (Table 2.9). The same reagents from different vendors are not always equivalent so the participating laboratories used the same reagents from the same vendors for the respective assays to reduce variability in the results.

The HCT-8 cells for the IFA cell culture infectivity method were grown in 8-well chamber slides. After inoculating and incubating the monolayers, the monolayers were stained for the presence of infectious stages. At the MWDSC laboratory, after fixing the monolayers with methanol, the chambers were removed from the slides for further processing. The coverslips were mounted with an anti-fade mounting medium and analyzed with an epifluorescence microscope. The AgriLife El Paso laboratory used an inverted phase contrast microscope equipped with epifluorescence to analyze the stained slides so the chambers were left intact on the slide and the staining was done in the individual chambers.

Controls

Infectivity Controls

A complete set of control assays was set up with every infectivity assay. The mock infection controls (25 oocysts inoculated onto the monolayer immediately before processing) were done to evaluate the potential background signal due to intact oocysts remaining on the monolayers without infecting. The trip control oocysts that were shipped along with the flow cytometry enumerated oocysts were inoculated onto monolayers at the time of infection. These positive control oocysts demonstrated that the infectivity of the flow cytometry sorted oocysts was not affected due to either the shipping process or the flow cytometry process. Gamma-irradiated oocysts (EasySeed, BTF) were also inoculated onto monolayers at the time of infection to show that the detection assay was not detecting a false positive signal. Uninoculated wells were also included in each experiment.

Table 2.9
List of common reagents

QA/QC For Cell Culture		
SAB-DEX plates	PML	P2300
TSA with 5% sheep blood	PML	P2600
BHI Broth	PML	T6243
Thioglycollate Broth	PML	T6480
Cell Culture Reagents		
HCT-8 cells	ATCC	CCL-244
1×PBS	Sigma	D8537
Trypsin-EDTA	Sigma	T4049
RPMI Medium 1640 (1×) with GLUTAMAX	Invitrogen	61870-036
Modified Eagle's Medium	Sigma	M2279
Fetal Bovine Serum 500ml-Heat Inactivated	Hyclone	SH30070.03-HI
4-aminobenzoic acid	Sigma-Aldrich	85,291-0
calcium pantothenate	Sigma	C8731
D (+)-Glucose	Sigma	G5400
Folic Acid	Sigma	F7876
Fungizone	Invitrogen	15290-018
HEPES (1M) 100mL	Invitrogen	15630-080
Kanamycin	Sigma	K0129
L-Ascorbic Acid	Sigma	A4544
L-Glutamine	Sigma	G7513
Penicillin-Streptomycin	Sigma	P0781
Tetracycline	Sigma	T7660
Nucleic Acid Extraction Reagents		
QIAamp DNA Mini Kit	Qiagen	51306
RNeasy 96 Kit	Qiagen	74181
ChargeSwitch Forensic DNA Purification Kit	Invitrogen	CS11200
Nucleic Acid Amplification Reagents		
Gene Amp 10× PCR Buffer (for PCR)	Applied Biosystems	N8080130
10×PCR Gold Buffer and $MgCl_2$ Kit (for PCR)	Applied Biosystems	4306898
Amplitaq Gold Polymerase (for PCR)	Applied Biosystems	4311816
dATP,dCTP,dGTP,dTTP (for PCR)	GE Healthcare Biosciences	27203501
dUTP (for PCR)	GE Healthcare Biosciences	27204001
BSA (for PCR)	NEB	B90015
Primers	IDT	
Uracil DNA Glycosylase (for PCR)	NEB	0280L
Platinum Taq, 10× Buffer and $MgCl_2$ (for RT-PCR)	Invitrogen	10966-034
dATP,dCTP,dGTP,dTTP (for RT-PCR)	Roche	11 969 064 001
dUTP (for RT-PCR)	Roche	11 934 554 001
Uracil DNA Glycosylase (for RT-PCR)	Roche	11 775 367 001
MuLV RT	Applied Biosystems	N8080018
Oligo $d(T)_{16}$ Primer	Applied Biosystems	N8080128
RNase Inhibitor	Applied Biosystems	N8080119
Staining Reagents		
Methanol	Sigma	M3641
1×PBS	Sigma	D8537
Goat Serum	Invitrogen	16210-064
Tween-20	Sigma	P9461
Rat anti-sporozotie antibody unlabeled	Waterborne	A600
Anti-rat IgG FITC labeled antibody	Sigma	F6258
Mounting medium	Waterborne	M101

Processing Controls

The RT-PCR reactions included both positive and negative controls. To check for the presence of DNA that may have carried over from the RNA extraction, a mock-RT reaction was run. This consisted of running an RT reaction with RNA and all the reagents for the reaction except the reverse transcriptase enzyme. The product of this reaction would then be used as the template in the PCR reaction. If any positive bands were generated, it would indicate the presence of carryover DNA in the RNA extraction, since there was no cDNA generated in the RT reaction (no RT enzyme present). Positive controls for the RT reaction consisted of extracted RNA that contained the target sequences. Positive controls for the PCR reaction was cDNA known to have the target sequence. These would both yield a positive band with gel electrophoresis. The PCR reaction positive control was DNA extracted directly from oocysts that were processed with the samples in the assay. A no template control was used for the negative controls for both the RT-PCR and PCR assays.

Evaluation of Free DNA in Flow Sorted Oocysts

The oocysts used in all of the experiments were initially stored and shipped as concentrated suspensions where there may be the possibility that a small percentage of the oocysts could excyst and the DNA could be released into the medium. This DNA could then theoretically be carried through the process of flow sorting and be placed into the sample tubes of oocysts that were inoculated onto monolayers. The extraneous DNA could then possibly be detected in the PCR based detection method. To show that this was not contributing to the higher than expected number of positives in the mock infection controls for the PCR based method, a set of samples of flow sorted oocysts (*C. parvum* and *C. hominis*) containing 3 and 10 oocysts/tube, respectively, in 150 µL was processed two different ways to show that there was no carryover of extraneous DNA. The first processing method included centrifugation of the samples and removal of 3 × 50 µL aliquots from each tube without disturbing the pellet of oocysts. The entire 50 µL sample was then used as template for a PCR reaction with hsp70 primers. In the second processing method, the sample was filtered through a 0.2 µm syringe filter into a fresh microfuge tube and the volume was brought up to 150 µL with water. The sample was then divided into 3 × 50 µL aliquots and the entire 50 µL sample was used as a template for the PCR reaction with hsp70 primers.

None of the samples prepared with centrifugation or filtration amplified. Therefore, any DNA detected in the mock infection samples was not from residual DNA in the oocyst preparations and must be from intact oocysts remaining on the monolayer.

Comparison of Three Cell Culture Infectivity Methods

Mock Infections

Mock infections were included in all assays to evaluate the potential background signal due to intact oocysts remaining on the monolayers rather than actual intracellular development stages. Wells were mock-infected with 25 oocysts (15 wells at each laboratory) and used for the three detection assays.

Table 2.10
Detection of low dose oocyst infectivity*

	Number of oocysts/well		IFA				
	Total	# Infectious oocysts†	% Pos. wells	% Infection (total # of oocysts)‡	% Infection (# infectious oocysts)§	PCR % Pos. wells	RT-PCR % Pos. wells
Viable oocysts (N=160 wells)	3	0.36	28.8 (N=160 wells)	12.1	97	51.2 (N=160 wells)	23.1 (N=160 wells)
Mock Infection	3	0.36	0 (N=160 wells)	0	0	17 (N=152 wells)	0.6 (N=160 wells)
Process Control (N=20 wells)	500	60.5	100	10.5	86.8	95	90
Gamma-irrad. (N=20 wells)	100	0	0	0	0	55	0

*Data compiled from 2 separate experiments, each experiment performed in duplicate at MWDSC and AgriLife El Paso.
†The number of infectious oocysts for every lot is calculated from the percentage of oocysts that formed infectious foci on HCT-8 cells in a preliminary QC test of each lot of oocysts.
‡Percent infectivity based on the total number of oocysts is the number of infectious foci detected on the monolayer divided by the total number of oocysts inoculated onto the monolayer (×100).
§Percent infectivity based on the number of infectious oocysts (determined by the QA infectivity assay performed on each lot of oocysts) is the number of infectious foci detected on the monolayer divided by the number of infectious oocysts inoculated onto the monolayer (×100).

Infectivity Detection With Low Dose Samples

To address the concern about positive results in the mock infection wells, two experiments were performed in which 40 wells were inoculated with three oocysts per well. This also tested the sensitivity of each method with low doses of oocysts. At the time of processing, 40 additional wells were "mock" infected with three oocysts. Mock infection entailed washing the monolayers, removing all medium from the wells, and then adding three oocysts (sorted by flow cytometry at WSLH in a volume of 10 µL) directly onto the monolayers. The wells were then immediately lysed (for the RT-PCR or PCR) or fixed (for the IFA method) and processed along with the other samples.

The results in Table 2.10 were compiled from two separate experiments, with each experiment being performed in duplicate at MWDSC and AgriLife El Paso. Results are shown as a percentage of wells positive for infectivity for each method. The results of the IFA method are also shown as percent infection based on the total number of oocysts seeded on the monolayer as well as percent infection based on the number of infectious oocysts seeded. The number of infectious oocysts is calculated from the percentage of oocysts that formed infectious foci on HCT-8 cells in a preliminary QA test of the oocysts.

The IFA method demonstrated good detection of infectivity. Nearly 30% of the wells were positive and the percent infectivity rate was 97% based on the total number of infectious oocysts inoculated onto the monolayer. No infectivity was detected for the mock infections or gamma-irradiated samples. For the PCR method, over 50% of the wells that were inoculated were positive

Table 2.11
Detection of infection in negative controls

	Frequency of positive wells:			
	Trial 1		Trial 2	
Controls	PCR	RT-PCR	PCR	RT-PCR
Unseeded (2 wells)	0%	0%	0%	0%
EasySeed (10 wells at 100 oocysts/well)	ND*	0%	ND	0%
Mock infection (6 wells at 100/well)	17%	0%	17%	17%
Heat inactivated (6 wells at 100/well)	ND	ND	100%	0%

*ND, not done.

but 17% of the mock infection wells were also positive. The RT-PCR method had 23% positive in the inoculated wells but had only 1 well (of 160) positive for the mock infections. The PCR method also had over 50% false positives in the wells that were inoculated with gamma-irradiated oocysts (although not directly related to environmental oocyst detection) while neither the IFA or RT-PCR methods generated false positive wells.

Therefore, the PCR method was the most sensitive but some of this sensitivity could be from false positive infections due to DNA present in the oocysts remaining on the monolayer. The RT-PCR method was not as sensitive but rarely had false positives. The IFA method was sensitive and did not produce false positives.

False Positives

An important criterion for choosing a method for the sampling phase of the project is that only oocysts that are infectious are detected. Non-infectious oocysts that may be present in the sample should not give a false positive result.

In a preliminary testing of the RT-PCR and PCR methods at MWDSC, negative control wells that were seeded with gamma-irradiated oocysts, heat inactivated oocysts and the mock infectious oocysts were analyzed for the presence of false positives. Table 2.11 shows that gamma-irradiated oocysts did not give a positive result for either method in either trial while the mock infection controls gave a positive result in both trials for the PCR method and in one trial for the RT-PCR method. Heat inactivated oocysts yielded false positive results in every well for the PCR method but no false positives for the RT-PCR method. This demonstrates that both the PCR and the RT-PCR methods are capable of yielding false positive results.

To rule out the possibility of bias, a blind study was performed to determine if the methods were capable of distinguishing between infectious and non-infectious oocysts. Aliquots of a fresh lot of infectious oocysts were rendered non-infectious by various methods (Table 2.12). These oocysts, along with untreated oocysts, were flow cytometry enumerated by WSLH and sorted into individual microcentrifuge tubes for inoculation onto cell monolayers. These oocysts were analyzed in all three cell culture infectivity assays at MWDSC and AgriLife El Paso. The Microbiology Laboratory at Orange County Utilities (OCU) analyzed a subset of samples using the IFA method only. The OCU results were comparable to MWDSC and AgriLife El Paso with 100% infectivity with 100 oocysts.

The treated oocysts were divided into two groups and processed separately. The first group included the oocysts that were inactivated by freeze/thawing and heat inactivating (Table 2.13). The second group included the oocysts inactivated by low pressure UV irradiation and gamma

Table 2.12
Methods used to inactivate oocysts

Inactivation method	Description
Heat inactivated	Heat oocysts to 70°C for 30 min., store at 4°C
Freeze/thaw	Place oocysts in liquid nitrogen for 5 min., transfer to 95°C heat block for 1 min., store at 4°C
UV treatment	Expose oocysts to approximately 60 mJ/cm^2 low pressure UV irradiation, store at 4°C
Gamma irradiation	EasySeed oocysts purchased from BTF (irradiation dose of 500 Gy), store at 4°C

Table 2.13
False-positive infections with inactivated oocysts

		Proportional infectivity					
		Live		Heat inactivated		Freeze/thaw	
		10*	100*	10*	100*	10*	100*
IFA	MWDSC	70†	100†	0	0	0	0
	AgriLife El Paso	60†	100†	0	0	0	20‡
PCR	MWDSC	30	50	10	0	0	10
	AgriLife El Paso	70	100	0	0	0	0
RT-PCR	MWDSC	30	100	0	0	0	0
	AgriLife El Paso	40	100	0	0	0	0

*Number of oocysts inoculated onto each monolayer.
†Average infectivity based on foci per inoculum oocysts = 9.9%.
‡Average infectivity based on foci per inoculum oocyst = 1%.

irradiation (Table 2.14). Untreated oocysts, infectious oocysts, process control oocysts, and mock infection oocysts were included in each group. To remove the possibility of bias in processing the samples, the infectious and inactivated samples were blind coded at WSLH and returned to the processing laboratories. The identity of the samples was only revealed after the final results were tabulated.

All the samples were processed using the standard AHBSS/T pretreatment prior to infection of the HCT-8 monolayers. All the samples from each group were processed at the same time and each laboratory processed the samples on the same day. After 64–72 hours days incubation at 37°C, the monolayers were processed according to the procedures described previously for each detection method. The results are summarized in Tables 2.13 and 2.14. Results are reported as proportional infectivity (the number of wells with a positive result divided by the total number of wells inoculated per dose × 100).

Positive results were detected with live oocysts in all three cell culture detection assays demonstrating that the detection methods were working properly. The RT-PCR method did not give any positive results for any of the inactivated oocysts while the IFA method was positive for the freeze/thaw inactivated samples in one laboratory. This positive result was at the high inoculation dose (100 oocysts/well) and only infected 2 of the 10 wells inoculated. While those false

Table 2.14
False-positives infections with UV and gamma-irradiated oocysts

		Proportional infectivity				
		Live		UV (~60 mJ/cm^2)		Gamma-irrad. (0.5 kGy)
		10*	100*	10*	100*	100*
IFA	MWDSC	90†	100†	0	0	0
	AgriLife El Paso	30†	100†	0	0	0
PCR	MWDSC	80	60	10	30	10
	AgriLife El Paso	80	100	40	90	100
RT-PCR	MWDSC	60	100	0	0	0
	AgriLife El Paso	50	100	0	0	0

*Number of oocysts inoculated onto each monolayer.
†Average infectivity based on foci per inoculum oocysts = 9.6%.

positives did not look like the typical infectious foci, they did meet the definition criteria for infection. The infectivity of these samples was very low. There was only 1 infectious focus per well, which equates to an average proportional infectivity of 1% of the inoculum dose. The average proportional infectivity of the controls was 9.9%. The infectious focus detected in these control wells could be the result of sporozoites released by an inactivated oocyst that were not capable of initiating an infection.

The PCR detection method had false positives with each type of inactivated oocysts, although they were at very low levels in the heat inactivated and freeze/thaw samples. This could be because the heating of the oocysts can damage the DNA so that even though it was present in the sample, it was not readily amplified. The UV and gamma-irradiation methods rendered the oocysts non-infectious, but did not damage the oocyst DNA to the extent that it was not amplifiable.

Troubleshooting the RT-PCR Method

Early in the project, problems arose with the RT-PCR method that indicated excessive DNA was being carried over during the RNA extraction procedure. Such carryover leads to false-positives with the RT-PCR method since amplification products are generated even if the target gene (hsp70) is not transcribed. The standard RNA extraction procedure (RNeasy-96, Qiagen) had worked consistently prior to this period. Therefore, considerable time was spent evaluating alternative RNA extraction methods with infected cell cultures (Table 2.15). At the end of these evaluations, it was determined that, with a second round of DNase treatment, the RNeasy-96 kit allowed for the most consistent and sensitive detection of infection. Since the larger volume of RNA was included in each RT-PCR reaction (to increase detection sensitivity), the DNase treatment was increased to ensure adequate removal of contaminating DNA. After the cell monolayers were lysed, the lysate was transferred to the membranes of the 96-well extraction plate (provided in the Qiagen kit). DNase 1 (18 KU/80 µL) was placed directly on the membrane and the extraction plate was incubated at 37°C for 20 min. The DNase 1 was removed by vacuum filtration and a second aliquot of DNase 1 was place on the membrane, the extraction plate incubated as before, and the DNase 1 removed. A comparison between one and two rounds of DNase digestion demonstrated

Table 2.15
Properties of commercial RNA extraction kits

	Extraction format:								
RNA extraction kit	Total RNA	mRNA	Oligo dT_{16} cellulose	Magnetic beads	Oligo dT hybridization	96 well format	Single tube format	Inclusive DNase incubation	DNase after extraction
Total Arrest RNA (Bioscience)	✓						✓		✓
Mag-Max 96 (Ambion)	✓			✓		✓		✓	
RNeasy-96 (Qiagen)	✓					✓		✓	
mRNA Catcher Plus (Invitrogen)		✓			✓	✓			✓
GenElute Direct (Sigma)		✓	✓				✓		✓
FastTrack Mag 96 (Invitrogen)		✓				✓		✓	
Micro-Fast Track 2.0 (Invitrogen)		✓	✓				✓		✓

reduced DNA carryover (reduced false-positives) but no detrimental effect on the sensitivity of detecting infection (88% infectivity with 25 oocysts, based on the proportion of wells that developed infection). Therefore, the standard extraction procedure with an additional DNase treatment was used for the remainder of Phase 1.

Limits of Detection

One of the criteria for choosing a method for the sampling phase of the project was that the method must be able to detect very low numbers of infectious oocysts. Infectivity trials with flow cytometry enumerated oocysts were conducted to determine the level of sensitivity of all three methods. Oocysts were sorted into individual tubes at concentrations of 1, 5, 10, and 25 oocysts per tube. Ten replicate monolayers for each oocyst dose were analyzed for each method. Each method also included the process control oocysts, gamma-irradiated oocysts, and wells with the mock infections. Table 2.16 shows that all three methods were able to detect infection with a single oocyst. Although there was some variability in the level of infectivity, this is to be expected since not every oocyst is infectious. Both the PCR and the RT-PCR method gave positive results for a few replicates of the mock infections while none of the methods showed infection for gamma-irradiated oocysts or in the unseeded wells. It is interesting to note that for the PCR method, false positives with gamma-irradiated oocysts were highly variable and in some experiments none were detected while in others all replicates were positive.

Data was combined from multiple infectivity trials with flow cytometry enumerated oocysts to determine the level of sensitivity of all three methods. All methods detected infection with a single flow cytometry enumerated oocyst, although not in all inoculated wells. The fact that a single oocyst does not produce an infection in every inoculated well is a reflection of the overall infectivity of each lot of oocysts. The average proportion of cell culture infectious oocysts, even in a fresh lot of oocysts is typically 5–10%. The average infectivity for each of the detection methods,

Table 2.16
Comparison of infectivity for three detection methods*

	% Infectivity (average)†					
	IFA		PCR		RT-PCR	
	MWDSC	AgriLife El Paso	MWDSC	AgriLife El Paso	MWDSC	AgriLife El Paso
	N=20 wells/dose	N=20 wells/dose	N=20 wells/dose	N=20 wells/dose	N=10 wells/dose	N=20 wells/dose
25	65	90	75	100	75	60
10	50	30	60	100	50	40
5	40	50	40	65	0	40
1	15	0	10	15	10	10
Mock (N=10)	0	0	0	20	ND‡	20
Trip Control (N=10)	100	100	100	100	ND	90
Gamma-Irrad. (N=4)	0	0	0	0	ND	0
Unseeded (N=10)	0	0	0	0	ND	0

*Two replicate experiments were performed in duplicate in each laboratory.
†% Infectivity based on the number of positive wells per number of wells inoculated.
‡ND, not done.

Table 2.17
Summary of detection of infection with a single oocyst

	% Infectivity (average)*	Standard deviation	Range	N†
RT-PCR	2.0%	5.5	0–10%	5
PCR	8.3%	7.5	0–20%	6
IFA	6.0%	13.4	0–30%	5

*Based on the number of positive wells per number of wells inoculated.
†Number of experiments.

based on the percentage of positive cell culture wells that were inoculated with a single oocyst, is presented in Table 2.17.

Detection of Cryptosporidium andersoni *Using the IFA Method*

Cryptosporidium andersoni originates from cattle and has frequently been found in environmental waters (Nichols et al. 2006; Ruecker et al. 2007; Yang et al. 2008). *C. andersoni* has also been shown to infect and develop in HCT-8 cell culture (Hijjawi et al. 2002; Wu et al. 2009), although intracellular developmental stages were viewed using light and electron microscopy rather than immunofluorescence microscopy. Therefore, it was initially uncertain whether *C. andersoni* would be detected using the cell culture IFA method developed for this project.

Oocysts of *C. andersoni* were purified from cow feces (supplied by Dr. Merle Olson, University of Calgary) using cesium chloride density gradient centrifugation by Norma Ruecker

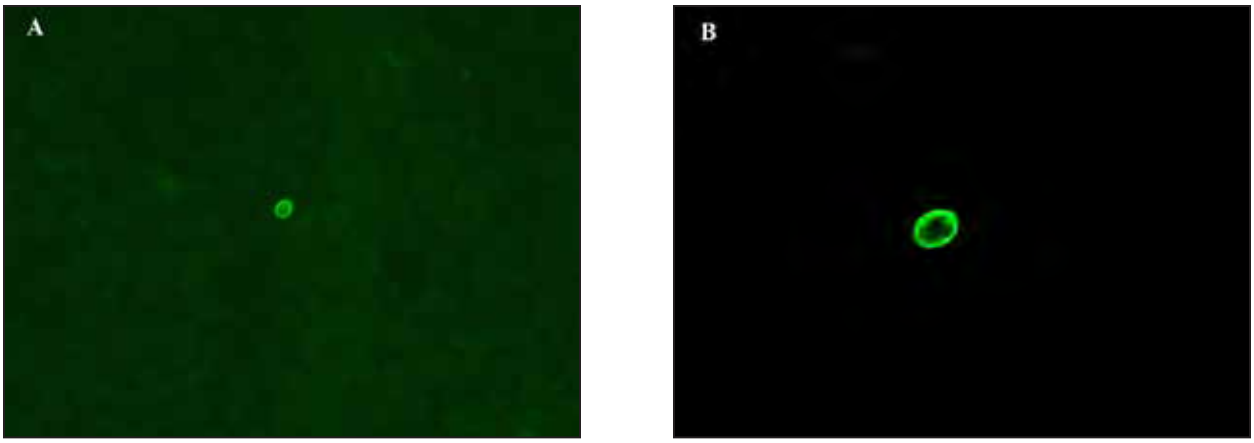


Figure 2.10 Immunofluorescence microscopy detection of a FITC-stained *Cryptosporidium andersoni* oocyst remaining on an HCT-8 monolayer. Monolayers were washed with 1× PBS and fixed with methanol prior to staining. Images were obtained at (A) 400× and (B) 1,000× magnification. Original images displayed bright green fluorescence.

(Provincial Laboratory for Public Health, Alberta). These oocysts were then enumerated by flow cytometry at WSLH. They were approximately two weeks post-shedding at the time of infection.

C. andersoni oocysts and *C. parvum* control oocysts were treated with AHBSS/T and replicate HCT-8 monolayers were inoculated with 1,000 and 100 *C. andersoni* oocysts. A *C. parvum* oocyst positive control and an uninoculated control were included. After 72 hours, monolayers were stained using the Waterborne SporoGlo primary antibody and FITC-labeled secondary antibody. In addition, one *C. andersoni* inoculated monolayer was also stained with EasyStain (BTF) to allow visualization of oocysts remaining on the monolayer.

No foci of infection were observed on the *C. andersoni* inoculated monolayers. The *C. parvum* positive control had typical foci of infection, and the uninoculated monolayer was negative. The monolayer stained with both Waterborne SporoGlo antibody and the BTF EasyStain had readily visible *C. andersoni* oocysts remaining on the monolayer (Figure 2.10). Some of the *C. andersoni* oocysts appeared intact while others appeared excysted. No *C. andersoni* sporozoites were observed on the monolayers.

It is possible that *C. andersoni* infected and developed in the HCT-8 cells. However, the SporoGlo antibody was developed using *C. parvum* sporozoite antigen and is unlikely to stain sporozoites or intracellular developmental stages of *Cryptosporidium* species other than *C. parvum*, *C. hominis*, and *C. meleagridis*. In this project we have clearly demonstrated that *C. parvum* and *C. hominis* are readily detected using the cell culture IFA protocol. Similar to the *C. andersoni* trial, we also tested *C. meleagridis* and *C. muris* in cell culture (10 day old TU1867 isolate, kindly provided by Dr. Saul Tzipori, Tufts University; and 1 day old RN66 isolate, Waterborne, Inc.). *C. meleagridis* has been previously shown to infect MDCK cell culture monolayers (Akiyoshi et al. 2003). In our study, infectious foci with staining intensity similar to *C. parvum* but containing fewer intracellular developmental stages were observed for *C. meleagridis* (Figure 2.11). No infectious foci were observed on monolayers inoculated with *C. muris*. These results suggest that only the major human infectious *Cryptosporidium* species (i.e. *C. parvum*, *C. hominis* and *C. meleagridis*) are detected using the HCT-8 cell culture-IFA method used for this project.

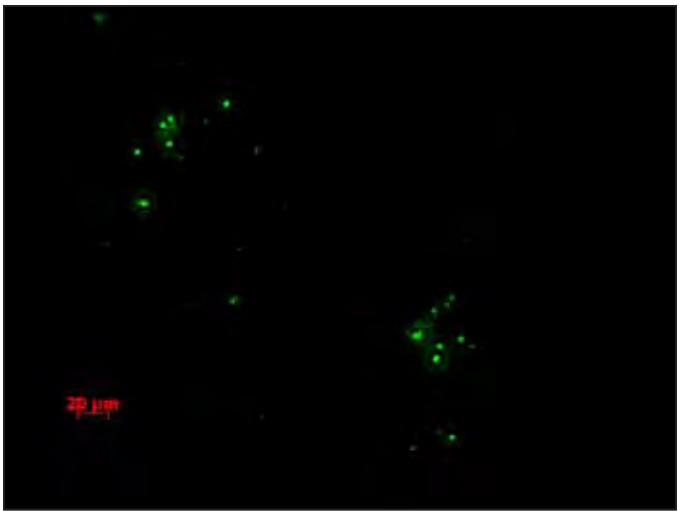


Figure 2.11 *C. meleagridis* TU1867 foci of infection in HCT-8 cells (200× magnification). Original image displayed bright green fluorescence.

Assessment of the Infectivity Methods on 1,000 L Spiked Filter Samples

The cell culture infectivity method selected for the large volume filtration studies in Phase 2 of the project had to be capable of detecting infectious oocysts from samples eluted from filters, therefore approximately 990 L of treated drinking water was filtered through 12 Envirochek HV filter capsules. In the laboratory, 10 L of drinking water spiked with five infectious oocysts (59 total oocysts, 11.8% infectivity) was filtered through each of six filters. Ten liters of unspiked water was filtered through the remaining six filters as blanks. The filters were eluted and the oocysts recovered by IMS. The recovered oocysts were treated with AHBSS/T to remove the magnetic beads and were then used to inoculate HCT-8 monolayers. Each laboratory processed three spiked and three unspiked filters.

As shown in Table 2.18, all three methods were able to detect infectious oocysts in the wells inoculated with the oocysts eluted from spiked filters. No infection was detected in the wells inoculated with the eluate from the unspiked filters. These data demonstrate that infectious oocysts could be eluted from 1,000 L drinking water samples filtered through Envirochek HV filter capsules and still retain their infectivity as detected by three different cell culture infectivity methods.

Genotyping of Positive Samples

The PCR and RT-PCR methods for detecting infection are completely compatible with genotyping since the cDNA or DNA that is end product of the extraction can be amplified by the sequencing primers. However, the IFA method required additional work to adapt the genotyping techniques to fixed monolayers that had been stained by the indirect fluorescent antibody procedure. Following fluorescence microscopy on the stained monolayers, DNA was extracted. Lysis solution was applied directly to the infectious focus and that area of the monolayer was then scraped off the slide using a sterile micropipette tip. The lysate was then transferred to a sterile microcentrifuge tube containing additional lysis buffer. The DNA was extracted (ChargeSwitch Forensic DNA Purification Kit, Invitrogen) and used as template for PCR amplification. Table 2.19 summarizes the source of DNA that can be used for genotyping positive samples.

Table 2.18
Infectivity of oocysts spiked onto filters with 1,000 L of drinking water and recovered by modified Method 1623*

	Number of oocysts/well			IFA			
	Total	# Infectious oocysts†	% Pos. wells	% Infection (total # of oocysts)‡	% Infection (# infectious oocysts)§	PCR % Pos. wells	RT-PCR % Pos. wells
Unspiked filter	0	0	0	0	0	0	0
Spiked filter	56	5	100	9	100	100	100
Infectivity	1	0.09	15	15	167**	15	10
Controls	5	0.45	40	12	133**	80	30
n=20	10	0.9	65	11	82	85	60
Process Control n=10	500	45	100	9	99	100	100
Gamma-irrad. n=10	100	9	0	0	0	10	0
Mock Infection n=10	25	2.25	0	0	0	10	0

*Data compiled from 2 separate experiments, each experiment performed in duplicate at MWDSC and AgriLife El Paso.
†The number of infectious oocysts for every lot is calculated from the percentage of oocysts that formed infectious foci on HCT-8 cells in a preliminary QC test of each lot of oocysts.
‡Percent infectivity based on the total number of oocysts is the number of infectious foci detected on the monolayer divided by the total number of oocysts inoculated onto the monolayer (×100).
§Percent infectivity based on the number of infectious oocysts (determined by the QA infectivity assay performed on each lot of oocysts) is the number of infectious foci detected on the monolayer divided by the number of infectious oocysts inoculated onto the monolayer (×100).
**Value can exceed 100% because the inoculum dose based on infectious oocysts is very low.

While the approach of fluorescent antibody staining followed by DNA extraction and PCR was straightforward, there were several potential obstacles. Since the monolayers had already been examined using epifluorescence microscopy, the effect of exposing the infectious foci to UV light on subsequent PCR detection was uncertain. It was possible that some of the *C. parvum* DNA in the infection foci may have been damaged, causing false negatives by PCR. An unfiltered 100 W mercury lamp delivers a UV dose of 1,000 mJ/cm^2 in a one second exposure on a fluorescence microscope (Severin and Ohnemus 1982). We used a Nikon Optiphot-2 microscope at 400× magnification and IL700 radiometer (International Light, Newburyport, MA) to measure the UV dose delivered to a sample during fluorescence microscopic observation. With a DAPI filter in place (330–380 nm excitation) samples received a UV dose of only 1×10^{-3} mJ/cm^2 during a 10 second exposure, which decreased to 2×10^{-4} mJ/cm^2 with the FITC filter (450–490 nm excitation). Therefore, it is unlikely that fluorescence microscopy induces sufficient DNA damage to inhibit PCR.

Comparison of Genotyping Primers

Two primer sets were tested for genotyping positive infectivity samples: the GP60 primers (Glaberman et al. 2002) and the 18S rRNA primers (Xiao et al. 2001). The 18S rRNA primer

Table 2.19
Source of DNA for genotyping

RT-PCR Infectivity	• cDNA from reverse transcription of RNA extracted from infected monolayers • Amplicon from RT-PCR
PCR Infectivity	• DNA extracted from infected monolayers • Amplicon from PCR
IFA Infectivity	• DNA extracted from infectious foci

	18S rRNA*	
	Forward primer (5'-3')	Reverse primer (5'-3')
Primary primers	TTCTAGAGCTAATACATGCG	CCCATTTCCTTCGAAACAGGA
C. parvum? from kangaroo, koala, black duck	..................A	.....
C. hominis	.....	..........C..........
C. suis	.....	..............T....
C. muris	.....	....T..........G.....
Nested primers	GGAAGGGTTGTATTTATTAGATAAAG	CTCATAAGGTGCTGAAGGAGTA
C. parvum? from dog, pig, kangaroo, ferret, *C. meleagridis*	....A.....	.....
C. hominis, C. canis	.............C.....	.....
C. felis	....A...C.....	.....

Figure 2.12 Specificity of 18S rRNA primers: The 18S rRNA primers have 100% similarity to the majority of *Cryptosporidium* spp. (*C. parvum, C. hominis, C. wrairi, C. meleagridis, C. muris, C. felis, C. canis, C. suis, C. serpentis, C. andersoni*) in GenBank with the exceptions indicated above

sequences have nearly 100% similarity to the majority of *Cryptosporidium* spp. in GenBank (Figure 2.12) so it follows that they would amplify most strains of *Cryptosporidium.* spp. if they were found in any of the samples. GP60 sequences are only available for *C. parvum* and *C. hominis* and many of these sequences were generated using the same or similar GP60 primers, so theoretical specificity cannot be assessed. Nevertheless, the GP60 locus has been shown to be highly discriminatory between strains within the *C. parvum* and *C. hominis* species. Since the species most likely to be found in treated drinking water samples that will be threat to public health are *C. parvum* and *C. hominis,* this should not be a problem.

The PCR template used for the genotyping primers varied depending on the cell culture detection method used. In order to demonstrate that the primers would work for each method, template from infections using each method was tested. For the RT-PCR method, cDNA from the reverse transcriptase reaction was used directly. In addition, the final PCR product following the RT-PCR detection reaction was used as a template. Theoretically, there should be enough of the original cDNA that was loaded into the reaction to be a target for the sequencing primers in the sequencing PCR. The same was done for the PCR method, both DNA directly extracted from the infected monolayers and the detection PCR products were tested with the sequencing primers. DNA was extracted from the fixed and stained infected cell monolayers for the IFA method. Table 2.19 summarized the source of DNA tested for both of the primers. The GP60 primers were

Table 2.20
Evaluation of genotyping primers with DNA from the three infectivity detection methods

Detection method	Type of DNA	Successful amplification by:* GP60	18S rRNA
IFA	DNA extracted from individual infectious focus	100%	100%
PCR	DNA extracted from infected monolayer	100%	100%
	Amplicon from PCR detection	50%	0%
RT-PCR	cDNA from reverse transcription of extracted RNA	100%	100%
	Amplicon from RT-PCR detection	100%	0%

*N = 4.

able to amplify DNA from all the sources tested while the 18S rRNA primers did not amplify DNA from the previously amplified samples of cDNA and DNA (Table 2.20). The 18S rRNA primers also produced false-positive bands in the negative controls (data not shown) due to cross-reactivity with the human genomic DNA present in the HCT-8 cells. Therefore, the GP60 primers were used for genotyping during Phase 2 of the study.

Distinguishing Between C. parvum *and* C. hominis *in Mixed Infections*

It was necessary to demonstrate that infectious foci resulting from infection with oocysts from different *Cryptosporidium* isolates or different species could be accurately distinguished from each other and the correct species of *Cryptosporidium* identified. Therefore, *C. hominis* (isolate TU728 obtained from G. Widmer of Tufts University) and *C. parvum* oocysts (mouse propagated Iowa isolate from Waterborne) were mixed and inoculated onto the same HCT-8 monolayers. Generally, *C. hominis* foci were smaller and did not stain as brightly as *C. parvum* foci although there were some foci of *C. hominis* infection that were larger than some *C. parvum* foci. Since the infectious foci are not adequately distinguishable by morphology (Figure 2.13), individual foci of infection were picked from the monolayer, and following lysis and DNA extraction, amplified by PCR using the GP60 primers, and the genotype determined by sequence analysis of the resulting amplicons. Individual infectious foci were readily genotyped by this method and *C. parvum* and *C. hominis* foci were differentiated in mixed infections. The results clearly demonstrated that individual strains or species of *Cryptosporidium* can be discerned in mixed infections (Table 2.21).

DISCUSSION

Various cell culture methods for assessing infectivity of *Cryptosporidium* have been described in the literature (see Table 1.1) but there has not been a study that directly compared these methods for their ability to detect infectious oocysts in large volume water samples. In order to determine the public health risk posed by the presence of *Cryptosporidium* oocysts in public drinking water systems, there must be some means of determining the infectivity of the oocysts to humans. It is important that the method used does not have a risk of detecting false positives and it would be helpful if it could also be able to identify the species involved.

This project compared three different cell culture infectivity methods with minor modifications to the published procedures. The RT-PCR method (Rochelle et al. 1997, 2002) analyzed RNA from infected HCT-8 cells, the PCR method (Di Giovanni et al. 1999, LeChevallier et al. 2003) analyzed DNA from infected HCT-8 cells, and the IFA method (Slifko et al. 1997, 1999) detected

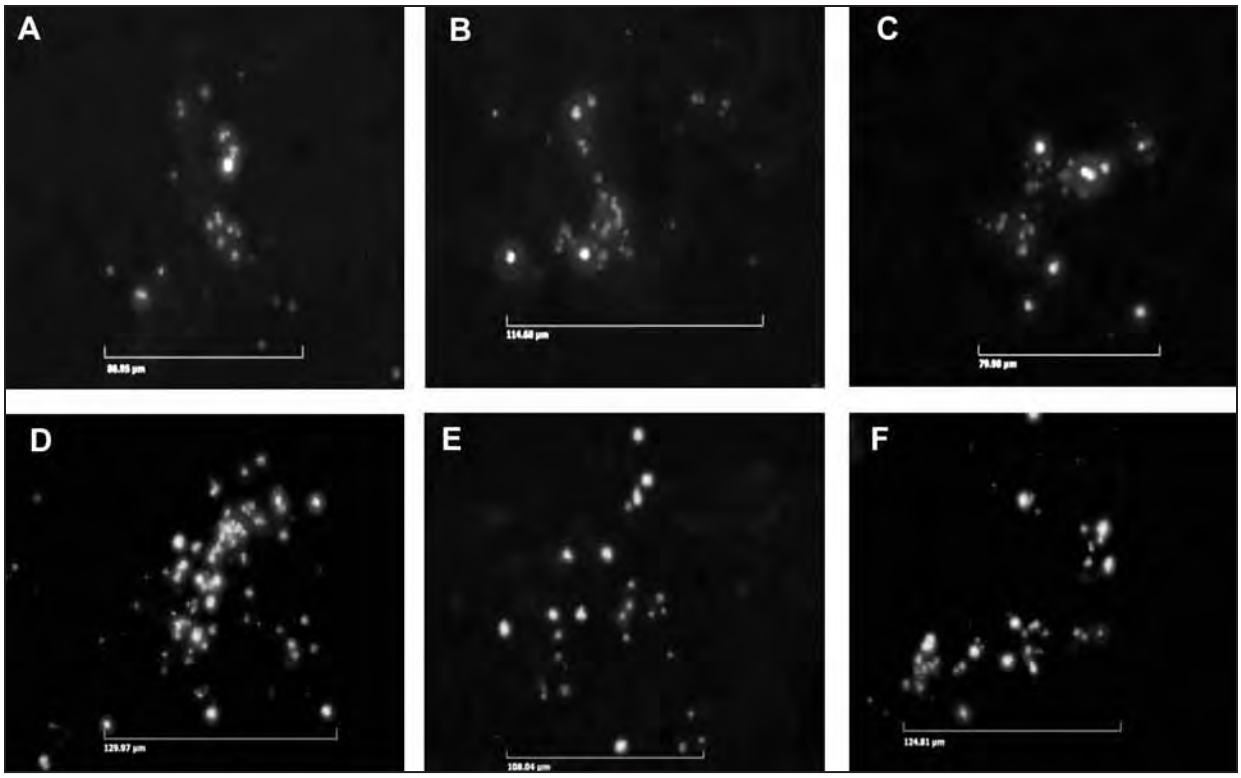


Figure 2.13 ***C. hominis*** **(A–C) and** ***C. parvum*** **(D–F) infectious foci in HCT-8 cell culture. Foci diameter in this figure range from 80 µm to 130 µm. Original images displayed bright green fluorescence.**

Table 2.21
Sequence analysis of IFA foci

Identity of oocysts	No. oocysts/well* (n=6)	No. foci/ well†	No. foci picked	No. samples amplified	Identity of foci
C. parvum	20	27.3	2	1	*C. parvum*
C. hominis	20	15.2	2	1	No amplification
C. parvum and *C. hominis*	10 *C. parvum* 10 *C. hominis*	15.3	10	5	*C. parvum* (4 samples) *C. hominis* (1 sample)

*Based on the number of infectious oocysts, total number of oocysts was much higher.
†Average of 6 wells.

infectious foci on HCT-8 monolayers using fluorescent antibody staining. The project compared sensitivity of detection, prevalence of false positives, variability of results, and ease of genotyping for the three methods.

Initially, the methods were tested for sensitivity of detection by inoculating the monolayers with 25, 10, 5, and 1 flow cytometry enumerated oocyst per well (see Table 2.16). All three methods were able to detect infection with a single oocyst but both the RNA and DNA methods also had positive results for the mock infection controls while the IFA method did not. The different

methods were then used to assess the rate of infectivity when inoculating the monolayers with three flow cytometry enumerated oocysts per well (see Table 2.10). A high number of samples (160) were processed alongside an equal number of mock infections of three oocysts per monolayer. This clearly demonstrated that although all the methods were capable of detecting the low numbers of infectious oocysts, only the IFA had no false positive results from the mock infection wells. The PCR method had the highest infectivity rate (51%) but also had the highest number of positive wells from the mock infections (17%) and the gamma-irradiated negative controls (55%) in the same experiment.

The ability of the methods to discriminate between infectious and non-infectious oocysts was assessed by inoculating HCT-8 monolayers with control oocysts that had been inactivated by a variety of methods (see Table 2.12) and then assessing infectivity with the three detection assays. No false-positive results were obtained with the RT-PCR method for any of these inactivated oocysts inoculated onto monolayers. Twenty percent of monolayers (N=10) assayed by IFA contained a single infectious focus when inoculated with 100 freeze/thaw inactivated oocysts. This equated to an average infectivity of only 1%, based on the number of foci per inoculum oocysts, compared to 10% for positive control samples (see Table 2.13). The PCR assay generated positive results for all the trials regardless of the method used to inactivate the oocysts. This was probably due to intact DNA being extracted and amplified even though the oocysts had been inactivated. However, an inoculation dose of 100 inactivated oocysts is much higher than would be expected in natural samples. Therefore, these controls represent worst-case scenarios and the actual rates of false-positives are likely to be lower for all of the assays. Consequently, the results with these control oocysts, treated to reduce their infectivity, provides confidence in the ability of the methods to discriminate between infectious and non-infectious oocysts.

Phase 2 of the project required that any samples generating a positive result be genotyped to determine the species or genotype of the infectious oocyst detected. Both the RT-PCR and PCR methods yield nucleic acid in the normal processing of the samples. The RT-PCR method only uses a portion of the extracted RNA so the surplus is available for the genotyping amplification reactions. The PCR protocol uses all of the extracted DNA in the amplification reaction but there was a sufficient amount of the original DNA in the final amplification reaction to be used as template for the genotyping amplification reactions. Since the IFA method does not extract nucleic acid as part of the detection method, subsequent processing needed to be performed to acquire DNA for genotyping. The infectious foci on the monolayer were scraped/lysed from the slide and the extracted DNA could be amplified for genotyping. The experiment that inoculated the HCT-8 monolayers with a mixture of *C. parvum* and *C. hominis* oocysts (see Table 2.21) demonstrated that the IFA method has the added benefit of analyzing an individual focus of infection for genotype. This provides additional information if a positive sample is the result of a mixture of several different species.

The IFA method was chosen as the most appropriate method to analyze the samples in Phase 2 of the study. The IFA method was sensitive in detecting low numbers of infectious oocysts, was able to differentiate infectious and non-infectious oocysts, and gave consistent results between the different trials as well as between the two processing laboratories. With the minor modifications mentioned previously to extract the DNA from the infected monolayers, the IFA method was compatible with genotyping positive samples. In addition, the IFA method was the only method that was capable of differentiating the species of an individual focus in a mixed infection.

CHAPTER 3
SURVEY OF TREATED DRINKING WATER FOR INFECTIOUS *CRYPTOSPORIDIUM*

INTRODUCTION

According to the only published study on the occurrence of infectious *Cryptosporidium* oocysts in conventionally filtered drinking water in the U.S., 27% of surface water treatment plants (N = 82) released infectious oocysts in their finished water at least once during a two-year monitoring period (Aboytes et al. 2004). Overall, 1.4% of treated drinking water samples (N = 1,690) contained infectious oocysts. Using the calculation in Equation 3.1, this occurrence figure translates to an annual risk of cryptosporidiosis of 52 infections per 10,000 people (U.S. national risk = 1.6 million cases per year), which is much higher than the 1 in 10,000 annual risk of infection goal set by the U.S. Environmental Protection Agency (USEPA).

$$\text{Annual Risk} = 1 - (1 - \text{Daily Risk})^{350} \tag{3.1}$$

$$\text{Daily Risk} = \text{water consumption} \times \text{concentration} \times \text{infection index}$$

where Water consumption = 1.2 L/day

Concentration in finished water = (number positive samples/total number samples) × (1/recovery efficiency) = 4.4×10^{-4} oocysts/L

Infection index = 0.028 for an unknown strain (according to Messner et al. 2001)

Reduction of sporadic cryptosporidiosis cases following installation of additional treatment demonstrated that drinking cold, unboiled tap water was a leading independent risk factor for infection (Goh et al. 2005). However, since many oocysts in surface waters may be inactive or belong to species other than *C. hominis* and *C. parvum*, the public health benefits of a risk assessment framework based solely on FITC-positive oocysts with no speciation, genotyping, or infectivity may be questioned.

Phase 2 of this project was designed to assess the occurrence of infectious *Cryptosporidium* oocysts in conventionally treated drinking water to determine whether the results of the earlier study could be replicated. The study cited above (Aboytes et al. 2004) used HCT-8 cells coupled with PCR to detect infectious oocysts. The same cell line was used for this project, but infections were detected using an indirect immunofluorescence assay (IFA), based on the results of method comparisons conducted in Phase 1 (Chapter 2). Desirable characteristics of an infectivity method for finished water include: distinguishing infectious from non-infectious oocysts; eliminating or minimizing false-positives and false-negatives; sensitivity to infection with environmentally relevant low oocyst numbers; robust enough to support infection despite the presence of environmental contaminants that are isolated along with the oocysts; and allow for molecular analysis of positive samples to determine the species or genotypes causing infection. The procedure selected at the end of Phase 1 (modified Method 1623 followed by in-vitro cell culture with detection of infection by IFA) met these criteria.

LARGE VOLUME FILTRATION

The total volume of water analyzed in the Aboytes et al. study was approximately 160,000 L (1,690 filters from 82 treatment plants). While the current project did not aim to replicate the same number of filters or treatment plants analyzed, a similar volume of water needed to be analyzed to allow a comparison of calculated risk based on the number of positive samples. Therefore, this project aimed to analyze fewer, large volume samples (up to 1,000 L) to ensure that at least 280,000 L were analyzed. Consequently, it was necessary to evaluate the recovery efficiency of Envirochek HV filters for large volume samples. The project eventually analyzed 349,053 L of finished water.

Large volume treated water samples (1,000 L) were spiked with 99 ± 1.7 (mean ± standard deviation) gamma irradiated oocysts (0.5 kGy, EasySeed; BTF, North Ryde, Australia). Due to the logistical difficulties in conducting numerous large volume (1,000 L) filtration recovery studies, recovery of oocysts with Envirochek HV filters was evaluated using three experimental procedures:

a. At MWDSC: Filtered 1,000 L of treatment plant effluent spiked with 99 EasySeed oocysts (10 × 100 L, each spiked with 100 µL of EasySeed [99 oocysts/mL], passed through a single filter)—2 filters.
b. At MWDSC: Filtered 800 L of finished water in the field (filter connected to a sample tap on the finished water reservoir, followed by 200 L of finished water spiked with 99 EasySeed (gamma irradiated) oocysts in the laboratory—6 filters.
c. At AgriLife El Paso: Filtered 990 L of unspiked tap water followed by 10 L of reagent water spiked with 100 EasySeed oocysts—2 filters.

Characteristics of treatment plant effluent for these samples were 0.06–0.07 NTU, 14–21°C, pH 7.93–8.57, 424–528 mg/L total dissolved solids, 2.03–2.60 mg/L chlorine residual. Filters were processed using a modified Method 1623 procedure. In all cases, the filters were treated with 5% sodium hexametaphosphate (w/v) to remove the mineral deposits that clog the filter and interfere with the elution of the oocysts (Clancy et al. 2003). The 5% sodium hexametaphosphate (HMP) solution was added to the filter and shaken at room temperature for 5 min. The HMP was immediately removed and clean reagent water was added to the filter, the filter was briefly shaken by hand and the water removed. The filter was then eluted and the eluant concentrated by centrifugation and IMS separation (per Method 1623). The bead-oocyst complex was dissociated in acidified Hanks Balanced Salt Solution/1% trypsin (AHBSS/T, pH 2.0) at 37°C for 1 hour. The oocysts were then placed on a well slide for staining. At MWDSC, oocysts were stained with an anti-*Cryptosporidium* FITC-conjugated antibody (Cellabs, Brookvale, Australia). MWDSC received USEPA tier 1 approval for this antibody in 2005. At AgriLife El Paso, recovered oocysts were also stained with the Cellabs antibody but the blank samples were stained with the EasyStain kit (BTF).

The average recovery efficiency for all 10 samples was 70.5% (range 44–90%; Table 3.1) which compares favorably with previous studies at MWDSC (mean = 59%, coefficient of variation = 21%, N = 6) and published reports (average = 62%, Clancy et al. 2003). Although much smaller volumes (10 L) of untreated water were used, it was demonstrated that Method 1623 recovery efficiencies for gamma-irradiated oocysts were not significantly different than viable, mouse-propagated oocysts (Francy et al. 2004). Both laboratories also filtered 1,000 L unspiked samples as blanks. All blanks were negative at MWDSC and AgriLife El Paso. At least 120 finished water

Table 3.1
Recovery of oocysts from 1,000 L finished water samples

Laboratory	Spiking procedure*	Recovery efficiency
MWDSC	1,000 L spiked	57%
MWDSC	1,000 L spiked	69%
MWDSC	800 L unspiked + 200 L spiked	44%
MWDSC	800 L unspiked + 200 L spiked	75%
MWDSC	800 L unspiked + 200 L spiked	62%
MWDSC	800 L unspiked + 200 L spiked	79%
MWDSC	800 L unspiked + 200 L spiked	69%
MWDSC	800 L unspiked + 200 L spiked	80%
AgriLife El Paso	990 L unspiked + 10 L spiked	90%
AgriLife El Paso	990 L unspiked + 10 L spiked	80%
	Mean	70.5
	standard deviation	13.4
	CV	19.0
MWDSC	1,000 L blank	0†
MWDSC	1,000 L blank	0†
AgriLife El Paso	1,000 L blank	0†
AgriLife El Paso	1,000 L blank	0†
AgriLife El Paso	1,000 L blank	0†

*Spikes were 99–100 flow cytometry enumerated EasySeed oocysts (BTF).
†Unspiked samples, so recovery efficiencies are not applicable. Number reflects total number of oocysts detected in 1,000 L of unspiked samples.

samples (200 L each) from this treatment plant have been analyzed by Method 1622/1623 over the last 10 years and neither *Giardia* nor *Cryptosporidium* have been detected.

Since the addition of sodium hexametaphosphate is necessary to ensure adequate recovery efficiencies from large volumes of finished water, its effect on oocyst infectivity was evaluated. Studies with oocysts incubated for 10 min at room temperature in 5% sodium hexametaphosphate, washed three times in 1× PBS, and inoculated onto HCT-8 monolayers demonstrated that it had no effect on oocyst infectivity (see Chapter 2).

RECOVERY EFFICIENCY OF *C. HOMINIS* BY MODIFIED METHOD 1623

Most of the method comparisons and evaluations were completed using *C. parvum*. Although it has been previously demonstrated that *C. hominis* infections can also be detected using cell culture and the various infection detection methods (see Chapter 2), the ability of the methods to recover and detect *C. hominis* was confirmed by additional experiments. Approximately 990 L of treated drinking water were filtered through six Envirochek HV filters. In the laboratory, 10 L of drinking water spiked with either 100 *C. hominis* oocysts (3 samples) or 100 *C. parvum* oocysts (2 samples) were then passed through the same filters. Ten liters of unspiked water was filtered through the last filter as a blank. Oocysts were also inoculated onto well slides (4 wells per dose) to determine the mean number of oocysts spiked onto the filters. The filters were eluted and the

Table 3.2
Recovery efficiency of *Cryptosporidium hominis* oocysts by modified Method 1623

	Recovery efficiency*
C. hominis –1	47%
–2	42%
–3	49%
C. parvum –1	100%†
–2	113%†
Blank	0%

*Number of recovered oocysts divided by the mean number of spiked oocysts × 100.
†Non-typical clumping observed in well slides used to determine oocyst dose.

oocysts recovered using a modified Method 1623. Magnetic beads were removed by acid dissociation and the samples put onto well slides (Superstick slides, Waterborne, Inc.) and allowed to dry. The samples were stained with anti-*Cryptosporidium*-FITC antibody (Cellabs) and counted. The average recovery efficiency for *C. hominis* oocysts was 46% (Table 3.2). There was non-typical clumping of the *C. parvum* oocysts on the well slides that led to an artificially low count for the number of oocysts spiked into the filters. This would explain the unusually high recovery efficiencies seen with the *C. parvum* samples.

PERFORMANCE EVALUATION OF ENVIROCHEK HV FILTERS

Ten cases of Envirochek HV filters (250 filters) were purchased for the survey phase of the project—five cases each from two lot numbers (A10644636, A10644417). MWDSC had three cases of each lot number while AgriLife El Paso had two cases of each lot. Oocyst recovery was determined for each lot of filters prior to their use in the project. The participating utilities either used filters from these lots or provided their own filters. If the participating utility provided their own filters, proper performance of the filters was determined by the utility. Recovery for the utility provided filters was 68.4% (N = 7, CV = 12.9%).

The mean oocyst recovery efficiency for 1,000 L spiked samples (990 L + 10 L spiked with 100 oocysts) by Method 1623 for these lots of filters was 68.5% (N = 4, CV = 20%), (Table 3.3).

UTILITY RECRUITMENT

A variety of methods was used to recruit utilities for the treated water survey phase of the project. Forty one utilities were contacted directly, a recruiting advertisement was placed on the Foundation's project profile website, announcements were made at the 2006 and 2007 AWWA Water Quality Technology Conferences, the PAC provided contact information, and referrals were requested from LT2ESWTR contract laboratories. Many utilities initially expressed interest in participating in the project, but many did not ultimately commit to the project because of uncertainty over the potential consequences of a positive result. Some utilities were hoping to use the results of the project in lieu of monitoring under the LT2ESWTR but once it became apparent that this would not be possible, they backed out of the project. Some utilities agreed to participate but their water was not suitable due to their treatment practices or, in one instance, only a limited volume

Table 3.3
Performance evaluation of specific lots of Envirochek HV capsules

Filter	Volume	Oocyst spike	Oocysts recovered
A10644636–1	1,000 L	100	53
A10644636–2	1,000 L	100	63
A10644417–1	1,000 L	100	73
A10644417–2	1,000 L	100	85
			Mean: 68.5% CV: 20%

of finished water (<100 L) could be passed through the Envirochek HV filters. A few utilities were interested in participating but were not able to filter samples on site.

The project eventually enrolled 14 utilities representing the southwest, northwest, midwest, and northeastern regions of the U.S. (USEPA Regions 3, 5, 6, 7, 8, 9, and 10). No utilities in the southeast were recruited for the project. A description of the participating utilities is presented in Table 3.4.

Cryptosporidium monitoring data was not available for the source water of all of the treatment plants used in this study and in some instances the data was over 10 years old. For those plants with available data (11 plants), oocyst concentrations were generally low (Table 3.5) so most plants would be classified in Bin 1 under the LT2ESWTR. However, at least two plants would probably be classified in Bin 2.

SAMPLE COLLECTION AND SHIPMENT OF FILTERS

Samples were filtered on site by utility personnel using filtration rigs provided by the project or their own equipment. An illustrated sample collection manual (Appendix A) was provided to all participating utilities. Filters were shipped to the two processing laboratories by overnight courier in coolers packed with blue ice.

SAMPLE PROCESSING

Upon receipt at the MWDSC and AgriLife El Paso laboratories, filters were processed using Method 1623 (USEPA 2005) modified for large volumes of finished water and to allow analysis by cell culture. A 5% sodium hexametaphosphate (HMP) solution was added to the filter and shaken at room temperature for 5 min. The HMP was immediately removed and clean reagent water was added to the filter, the filter was briefly shaken by hand and the water removed. Filters were then eluted and the eluate concentrated by centrifugation and IMS (per Method 1623). The bead-oocyst complex was dissociated in acidified Hanks Balanced Salt Solution/1% trypsin (AHBSS/T, pH 2.0) at 37°C for 1 hour. After two washes with fresh cell culture media to remove any traces of trypsin, the dissociated oocysts were inoculated onto confluent HCT-8 cell monolayers on 8-well slides. Cells were incubated for 64–72 hours and following methanol fixation, slides were stained with SporoGlo anti-sporozoite antibody (Waterborne, Inc.) and observed using epifluorescence microscopy.

Table 3.4
Description of treatment plants

Utility ID	Age* (years)	Total capacity (MGD)	Treatment processes	Source water	Population served
P1	92	150	Conventional—potassium permanganate, chlorine disinfection, flocculation, sedimentation, filtration, chlorine for dual media residual disinfection	Lake	~340,000
P2	59	28	Conventional treatment—Pretreatment with 3-stage flocculation and lamella inclined plate settlers; filtration with GAC media; disinfection with free chlorine, chloramines for residual	River	48,000
P3	66	40	Conventional treatment with chlorine dioxide used as a pre-disinfectant	River	~300,000
P4	16	60	Raw water ozonation, coagulation, settling, recarbonation, pre-filter ozonation, monomedia GAC filtration and breakpoint chlorination	River	~300,000
P5	105	220	Conventional treatment—Coagulation, flocculation, sedimentation. Rapid sand filtration, GAC filtration, disinfection with chlorine gas.	River	800,000
P6	na†	56	Conventional—potassium permanganate, chlorine disinfection, flocculation, sedimentation, filtration, chlorine or chloramine disinfection (depending on temperature)	River	~150,000
P7	106	30	Conventional treatment—Coagulation and settling processes and parallel Superpulsator clarification, followed by GAC filtration	River	135,000
P8	68	520	Conventional treatment—Disinfection, coagulation, flocculation, sedimentation, filtration. Chloramination for residual disinfection	River	2,301,000
P9	99	140	Presedimentation with $KMnO_4$; flocculation with NaOCl/PAC, ferric chloride/polymer, and lime applied between pre-sedimentation and flocculation	River	528,000
P10	50	220	Water shed management, screening, chlorination, chloramination, pH adjustment	River	860,000
P11	25	54	Conventional treatment—chemical pretreatment for coagulation, flocculation, and sedimentation prior to sand filtration and final chlorine disinfection.	River	235,000
P12	38	144	Ozonation, coagulation, flocculation, filtration, disinfection, chloramination	Lake, rivers	>1,000,000
P13	44	160	Coagulation, flocculation, filtration, disinfection, chloramination	Lake, rivers	>1,000,000
P14	~100	96	Coagulation, flocculation, sedimentation and filtration and chlorine for disinfection	Rivers, streams,	~600,000

*The age of treatment plants is based on their date of construction but most have been modified and upgraded since construction.
†na, data not available.

Table 3.5
Cryptosporidium* in source water of utilities that supplied water

Oocyst concentration	Monitoring period
0.2 oocysts/L	April 2008 to March 2009
0.06 oocysts/L	January 2007 to January 2008
0.15 oocysts/L	January 2007 to January 2008
<0.001 oocysts/L	10 years of monitoring
None detected	2006 to 2009
0.08/L	2 years of monitoring data
None detected	2002 to 2009
0.02 oocysts/L	3 yrs of monitoring data
0.0017 oocysts/L	1998
0.0027 oocysts/L	1998

*Information not available for all treatment plants.

TREATED WATER SURVEY

Fourteen treatment plants across the U.S. participated in the survey of treated waters. Characteristics of these water samples are provided in Table 3.6. A total of 370 samples was analyzed by the two laboratories. Sample volumes ranged from 83.5 to 2,282 L with an average of 943 L for a total volume of 349,053 L. The volume of water filtered for each sample depended on water quality characteristics, the amount of water that passed through the filter before it clogged, problems with individual filtration rigs (e.g., inadequate pressure or flow control), or operational issues at the treatment plant. Nevertheless, 90% of samples were >600 L and 82% were >900 L. Therefore, the majority of samples fit the definition of "large volume."

None of the 370 finished water samples analyzed for this project produced infections that were detected by the cell culture/IFA assay. Control infections that were run in parallel with each set of samples demonstrated that the procedures and assay were working within expected criteria. Explanations and consequences of this zero result are discussed below.

CONTROL INFECTIONS AND MATRIX SPIKES

This study was not designed to directly compare results from the two analytical laboratories. However, comparison of QC infectivity controls, positive control infections, and matrix spikes illustrates the general reproducibility and robustness of the method. Intra- and inter-laboratory variability was observed but a certain level of variability is inherent in the method since it involves multiple living systems and many factors that influence performance. Since both laboratories were experienced with all of the methods and protocols, and there was considerable inter-laboratory technology transfer and communication during Phase 1 of the project, it is unlikely that differences in laboratory technique were a major contributor to this variability.

Freshly shed oocysts (*C. parvum* Iowa isolate) were obtained regularly throughout Phase 2 of the project to use in matrix spikes and positive infection controls. All oocyst lots were thoroughly assessed using the QA/QC procedures described in Chapter 2. Infectivity QC involved inoculating six replicate cell culture wells with 1,000 IFA-enumerated oocysts and processing by cell culture-IFA. Infectivity was expressed as the number of infectious foci as a percentage of the inoculum size (1,000 oocysts). The mean QC infectivity for oocysts used during Phase 2

Table 3.6
Characteristics of treated water samples analyzed for infectious *Cryptosporidium*

Utility	Number of samples processed at: MWD	EP	Total volume (L)	Turbidity (NTU) Influent*	Effluent†	pH	TDS (mg/L)	Temp. (°C)	Cl_2 (mg/L)
P1	12	6	20,947	1.1–611	0.05–0.07	7.1–7.3	na	1–23.3	1.05–1.45
P2	4	4	7,043	1.05–157	0.05–0.16	7.5–8.1	305–466	5–14.5	1.36–1.9
P3	13	14	27,011	na	0.04–0.1	na	na	na	1–2.32
P4	16	14	29,558	na	0.04–0.1	6–7.1	120–773	na	0–2
P5	8	8	15,210	1.5–107	0.04–0.08	7.3–8.7	na	3–27	na
P6	19	19	38,401	20–215	0.05–0.14	7.2–7.6	136–517	2.2–27.8	2.5–4
P7	18	20	38,432	4–143	0.04–0.13	7.1–7.6	201–257	0.8–23.3	0–3.79
P8	23	21	44,889	0.3–2.1	0.05–0.21	8.0–8.3	334–545	11–26	2.29–2.63
P9	7	8	15,489	1.8–31.4	0.05–1.93	7.0–7.2	na	5–27	1.9–2.3
P10	16	16	6,277	na	0.23–3.09	7.0–8.0	na	3.8–15.2	0.61–1.67
P11	20	23	46,606	na	0.02–0.49	7.1–7.9	na	na	0.01–1.64
P12	12	11	23,000	0.3–0.8	0.08–0.18	8.3–9.6	78–116	9–20	1.86–2.5
P13	14	9	21,000	0.5–12.7	0.04–0.29	7.2–9	140–237	9–18	1–1.9
P14	8	7	15,191	3.8–24	0.08–0.16	7.4–8.0	na	3.3–24	1.7–2.46
Total	190	180	349,053						

*Turbidity of raw water coming into the filtration plant.
†Turbidity of treated water as it leaves the filtration plant.
MWD, Metropolitan Water District of Southern California.
EP, Agrilife El Paso.
na, Data not available.

was 10.8% (coefficient of variation = 34.4%, N = 163). Infectivity was never 100% (or anywhere approaching 100%) because even in a freshly shed population of oocysts, only a small portion of the oocysts are capable of initiating an infection (typically 5–15%).

To assess recovery efficiencies and the effect of the different matrices on the infectivity assays, water samples from each utility were filtered and the filters shipped to each analytical laboratory for spiking (Table 3.7). At each laboratory, an additional 10 L of reagent water was spiked with either 500 IFA enumerated viable *C. parvum* oocysts (mouse propagated Iowa isolate from Waterborne, Inc.) or 100 flow cytometry enumerated ColorSeed (gamma-irradiated) oocysts (BTF) and filtered through the capsules provided by each utility. IFA enumeration of oocyst spikes was based on 10 replicate well counts of stained oocyst suspensions. Oocyst preparations were only used for spiking studies if their QC infectivity was at least 5%.

Filters spiked with ColorSeed oocysts were processed using the sodium hexametaphosphate modified Method 1623 procedure and recovered oocysts were deposited directly onto well slides and enumerated by epifluorescence microscopy. Recovery efficiency was calculated as the number of observed oocysts as a percentage of the 100 spiked oocysts. Filters spiked with fresh (viable) Iowa oocysts were processed using the modified Method 1623 procedure and inoculated onto a cell culture monolayer. For all matrix spike samples, the positive control was two replicate cell culture wells inoculated with 500 IFA enumerated oocysts.

Table 3.7
Matrix spike recovery efficiencies

			Infectivity*				ColorSeed†	
Utility	Date	Analysis Lab.	Volume (L)	Matrix spike foci	Control infection foci‡	Recovery (%)	Vol. (L)	Recovery (%)
P1	8/22/2008	MWDSC	1003	50	25	200	1003	50
P1	8/22/2008	EP§	1003	28	67.5	85.9		
P2	1/30/2009	MWDSC	945	96	50	192	945	5
P2	1/30/2009	EP	1000	96	90.5	106.1	1000	54
P2	5/7/2009	EP					998	79
P2	5/8/2009	MWDSC	999	68	67.5	100.7		
P3	8/31/2007	MWDSC	759	37	61.5	60.1	592	62
P3	3/7/2008	MWDSC	907	106	120	88.1	718	20
P3	3/7/2008	EP	394	71	86	82.6	577	54
P4	8/31/2007	EP	1629	5	83	6	1778	32
P4	3/21/2008	MWDSC	916	38	37	100		
P4	3/21/2008	EP	1071	36	65.5	55	903	40
P5	6/13/2008	MWDSC	648	11	62	17.7	586	19
P5	6/13/2008	EP					590	48
P5	9/12/2008	EP	1548	16	30	53.3		
P5	4/24/2009	MWDSC	852	94	89	100.6		
P5	5/7/2009	EP					953	86
P5	5/8/2009	MWDSC	1045	66	67.5	97.8		
P6	11/16/2007	MWDSC	1000	99	108	92		
P6	11/16/2007	EP	1000	85	95	89.5		
P6	12/5/2008	MWDSC					1002	31
P6	12/5/2008	EP					1002	22
P6	12/19/2008	MWDSC	1002	17	45	37.8		
P6	12/19/2008	EP	1002	73	85.5	85.4		
P7	10/5/2007	EP	1057	38	69.5	54.2		
P7	10/8/2007	MWDSC	1000	79	56.5	139	1008	77
P7	11/16/2007	EP					1001	16
P7	12/5/2008	MWDSC					1000	53
P7	12/5/2008	EP					1000	69
P7	12/19/2008	MWDSC	1000	43	45	95.6		
P7	12/19/2008	EP	1000	63	85.5	73.7		
P8	6/29/2007	MWDSC	990	80	82	97.6	995	42
P8	6/29/2007	MWDSC	991	13	82	15.8	991	53
P8	6/29/2007	EP	1063	106	99	107	991	40
P8	6/29/2007	EP	991	100	99	100	1022	35
P8	8/31/2007	EP	1013	31	83	37.3		
P8	12/17/2007	MWDSC					1007	26
P8	12/17/2007	EP	1003	77	71	92.2		

(continued)

Table 3.7 (Continued)

			Infectivity*				ColorSeed†	
Utility	Date	Analysis Lab.	Volume (L)	Matrix spike foci	Control infection foci‡	Recovery (%)	Vol. (L)	Recovery (%)
P9	2/22/2008	MWDSC	2024	87	105.5	82.5	2489	21
P9	2/22/2008	EP	2142	14	77	18.2	2373	9
P9	3/7/2008	MWDSC	1011	76	120.3	63.2		
P9	3/7/2008	EP	1003	44	86	51.2	1002	2
P9	3/7/2008	EP					1084	29
P9	12/5/2008	MWDSC					1003	49
P9	12/5/2008	EP					1002	38
P10	6/27/2008	MWDSC	300	2	29	6.9	300	35
P10	6/27/2008	EP	300	14	47.5	29.5	300	8
P10	8/22/2008	EP					151	10
P10	5/7/2009	EP					300	20
P10	5/8/2009	MWDSC	300	63	67.5	93.3		
P11	8/31/2007	MWDSC	2051	2	61.5	3.1		
P11	8/31/2007	MWDSC	1010	41	61.5	67		
P11	8/31/2007	EP	1206	17	83	20.5	1097	3
P11	8/31/2007	EP	1396	73	83	88	852	60
P11	12/5/2008	MWDSC					606	34
P11	12/5/2008	EP					878	74
P11	12/19/2008	MWDSC	994	31	45	68.9		
P11	12/19/2008	EP	1061	41	85.5	48		
P12	9/12/2008	MWDSC	990	45	42	107.1		
P12	9/12/2008	EP	990	49	30	163.3	990	76
P13	8/31/2007	MWDSC					2309	14
P13	8/31/2007	MWDSC					1310	62
P13	9/26/2008	MWDSC	600	94	121.5	77.6	600	52
P13	10/10/2008	EP	1000	101	80.5	125.5	1000	50
P13	5/7/2009	EP					1000	71
P13	5/8/2009	MWDSC	1000	47	67.5	69.6		
P14	3/7/2008	MWDSC	915	105	120.3	87.3	921	40
P14	3/7/2008	EP	921	102	86	118.6	915	57
P14	12/19/2008	EP	989	98	85.5	114.6		
P14	5/8/2009	MWDSC	989	68	70	100.7	989	93
Mean			1020	57.6	73.8	79.8	981	41.7
SD			363	32.6	24.6	42.7	468	23.8

*Recovery efficiency based on number of infectious foci from filter spiked with 500 oocysts relative to number of infectious foci from control infection with 500 oocysts.
†Recovery efficiency based on number of ColorSeed oocysts detected on monolayer after spiking 100 oocysts into filter.
‡Average of duplicates monolayers each seeded with 500 Iowa oocysts (N = 2 for most positive controls but in a few instances N = 4 or 6).
§EP, AgriLife El Paso.

The infectivity recovery efficiency for the spiked filters was calculated as the number of infectious foci as a percentage of the number of infectious foci in the positive control infections. For example, if 40 infectious foci developed in the positive control infections and 20 were observed for the matrix spike, the recovery efficiency was expressed as 50%. Due to logistics at the utilities and each of the analytical laboratories, ColorSeed and infectivity recovery efficiencies were not necessarily determined on samples collected on the same day and not all matrix spikes were analyzed by both laboratories. Nevertheless, 3–11 matrix spikes were analyzed for all utilities, matrix spikes from all utilities were processed by both laboratories, and ColorSeed and infectivity matrix spikes were processed on the same day for all except one utility (P6). Sample volumes for matrix spikes were 300–2,373 L (mean = 992 L, N = 97).

There was no significant difference between the two laboratories' results for positive control infections (means = 13.4% and 14.5%, $P = 0.21$) and there was generally good agreement in direct sample-sample comparisons (Figure 3.1). The average infectivity for both laboratories combined was 13.9% (range, 4.6–28.6%, N = 141). These results indicate that inter-laboratory variation was no greater than intra-laboratory variation, demonstrating the robustness of the method. With combined data from both laboratories, infectivity in the positive controls (500 inoculum oocysts, mean infectivity = 13.9%) was significantly higher ($P < 0.001$) than in the initial QC infections that were inoculated with 1,000 oocysts (mean infectivity = 10.8%). This was possibly due to overlapping foci of infection being counted as single foci on slides inoculated with the higher number of oocysts.

Infectivity recoveries from matrix spikes were 3–200% (mean = 84%) at MWDSC and 6–163% (mean = 75%) at AgriLife El Paso. There were no significant differences between the two analytical laboratories for ColorSeed or infectivity recovery efficiencies (oneway analysis of variance: ColorSeed $P = 0.65$, infectivity $P = 0.46$, 95% confidence level). An infectivity recovery efficiency of 100% means that 500 oocysts, spiked into a filter and processed through the Method 1623 and cell culture procedures, generated the same number of infectious foci (detected by immunofluorescence microscopy) as 500 oocysts that were inoculated directly onto a cell monolayer. The results from spiked samples processed in both laboratories demonstrated the relative robustness of the method.

There was not a strong sample-to-sample correlation between the two laboratories for matrix spike recovery efficiencies for either ColorSeed or infectious oocysts (Figure 3.2). For example, in one instance MWDSC reported a ColorSeed recovery of 5% compared to 54% at AgriLife El Paso. Similarly, for another sample, infectivity recovery at MWDSC was 83% compared to 18% at AgriLife El Paso. ColorSeed recoveries were based on the number of flow cytometry enumerated oocysts spiked into the matrix and so they represent absolute recoveries of these oocysts. Since only a portion of a population of freshly shed oocysts is able to initiate an infections (typically 5–15%), infectivity recoveries were based on the number of infectious foci from the matrix spiked with 500 oocysts expressed as a percentage of the number of foci that developed on a monolayer inoculated directly with 500 of the same control oocysts. Consequently, the calculated infectivity recovery efficiency was sensitive to both the number of foci from the matrix spike and the control infection. For example, the matrix spikes conducted on 1/30/09 for utility P2 resulted in widely different recoveries of 192% and 106% at MWDSC and AgriLife El Paso, respectively (Table 3.3). However, both laboratories detected the same number of infectious foci (96) on the monolayers inoculated with oocysts recovered from the matrix spike samples. The difference in recovery efficiency between the two laboratories was due to the difference in the number of infectious foci on

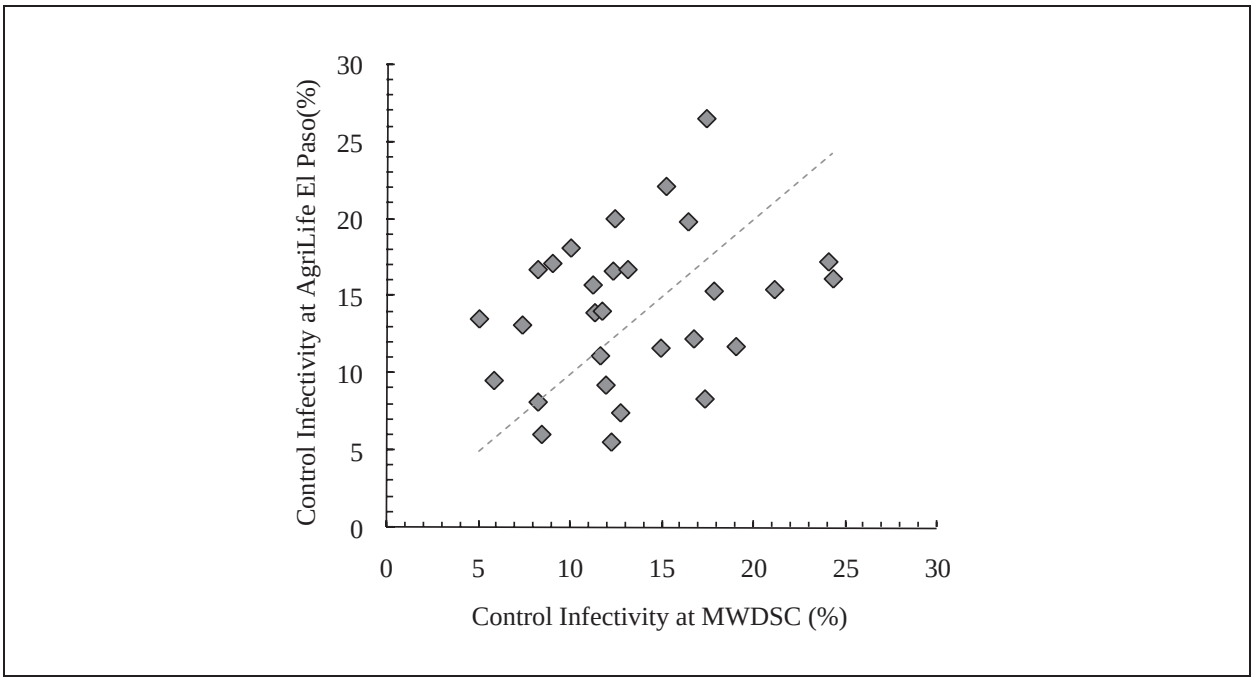


Figure 3.1 Correlation between the two analytical laboratories for positive control infections (inoculated with 500 oocysts) processed alongside matrix spike samples. The dashed line indicates the perfect correlation.

the control monolayers in each laboratory. Figure 3.3 displays the correlation between the two laboratories for matrix spike infectivity based solely on the number of infectious foci.

With the exception of two possible outliers that generated unusually high infectivity recovery efficiencies, there was a weak but statistically significant correlation between ColorSeed and infectivity recoveries when data from both laboratories were combined (Figure 3.4; $R^2 = 0.34$, $0.01>P<0.05$). This indicates that low infectivity recoveries were probably due to oocyst loss during sample processing rather than matrix interference with oocyst infectivity. There was no correlation between recovery efficiencies and sample volume (Figure 3.5) and low recovery efficiencies were not linked to any particular lot number of Envirochek HV filters.

Freshly shed oocysts were also spiked into 10 L of reagent water every few weeks throughout Phase 2 and processed using the modified Method 1623-cell culture-IFA procedure as a measure of the ongoing precision and recovery (OPR). For the matrix spikes, recovery efficiency was calculated as the number of infectious foci from the OPR samples as a percentage of the infectious foci generated by the 500-oocyst positive control infections. The average infectivity recovery efficiency for OPR samples conducted over two years was 75.9% (CV =37.2%, N = 33), which is very close to the value obtained from matrix spikes (mean = 79.8%). However, for OPR samples that were processed on the same day as matrix spike samples, there was no significant correlation between OPR and matrix spike recovery efficiencies (Figure 3.6; $r = 0.34$, $P>0.05$, N = 24).

BLIND RECOVERY STUDY

About half-way through the survey phase of the study after processing 144,608 liters of finished water from eight different utilities and not detecting any positive samples, it was necessary to reaffirm that the elution method and IFA cell culture detection method being used was capable

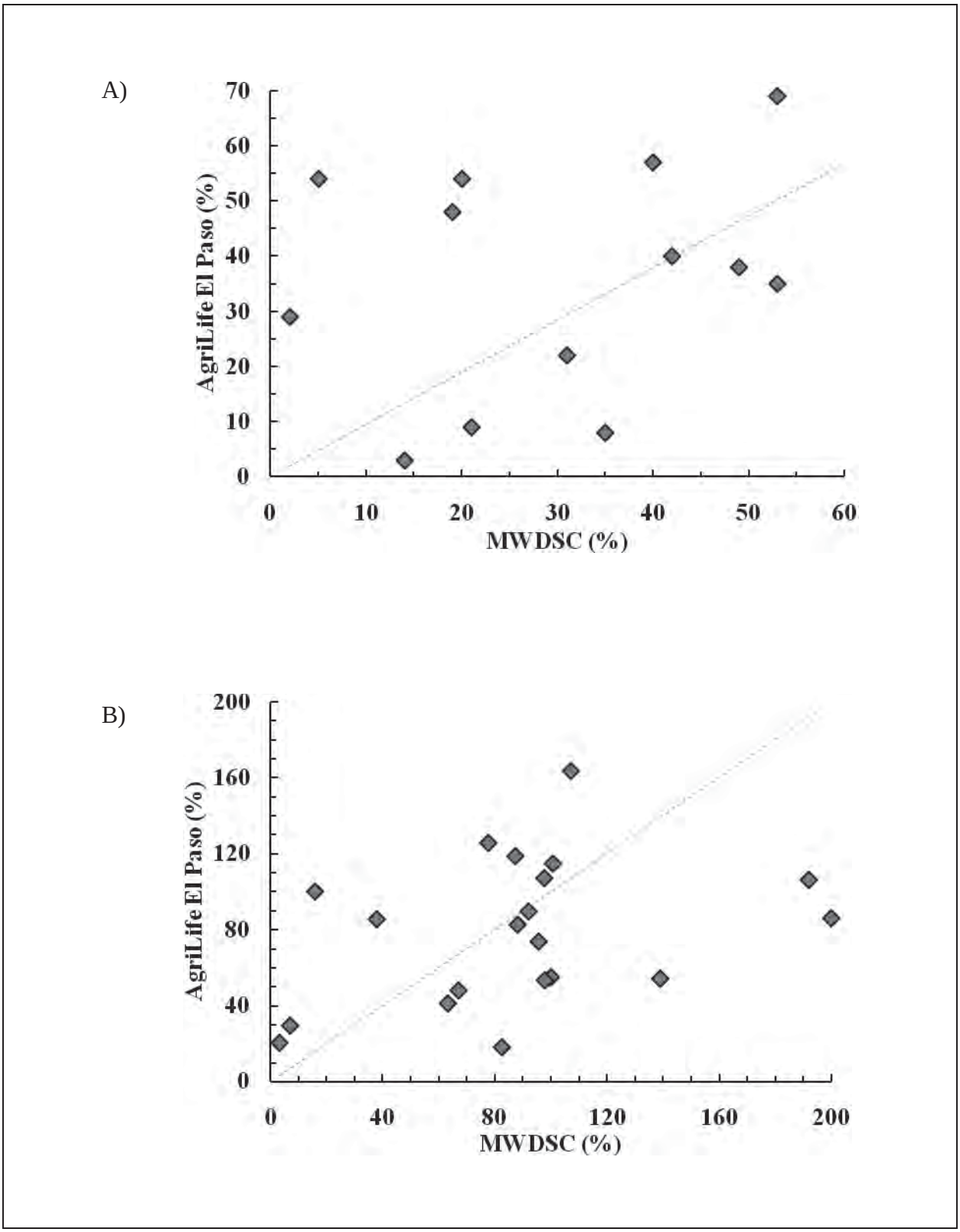


Figure 3.2 Comparison between the two analytical laboratories for ColorSeed (A) and infectivity (B) recovery efficiencies in spiked matrix samples from each of the utilities. The dashed lines indicate perfect correlations.

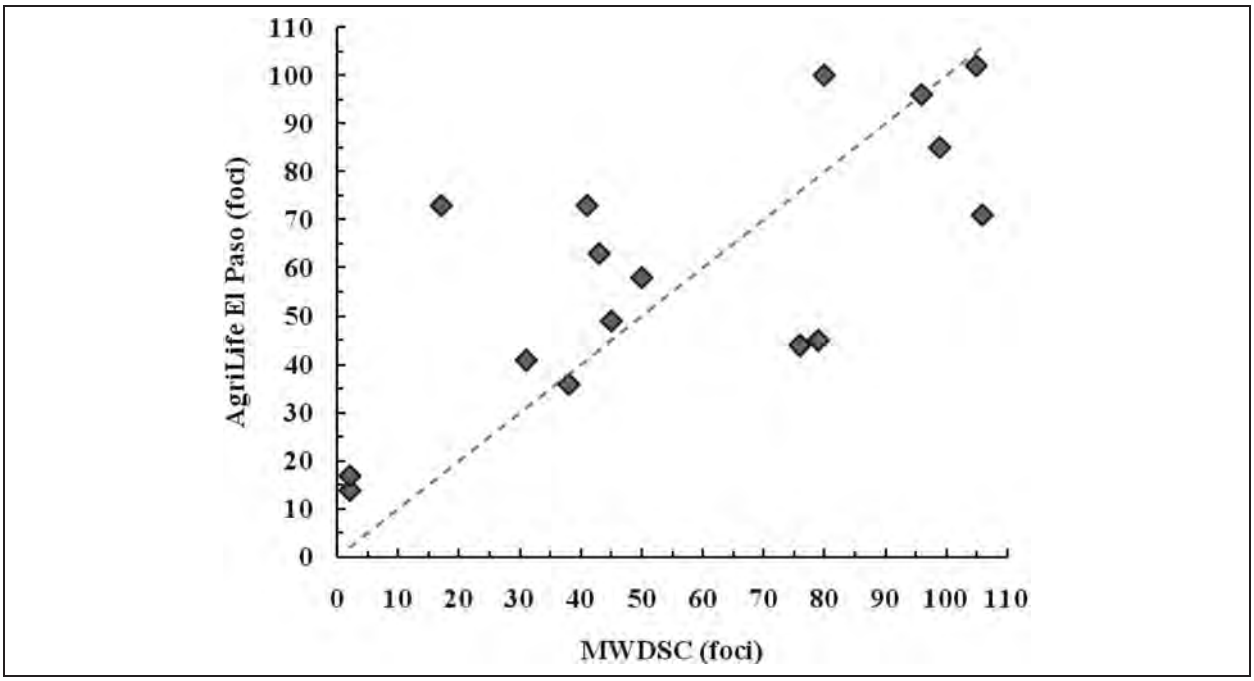


Figure 3.3 Comparison between the two analytical laboratories for the number of infectious foci that developed on monolayers inoculated with oocysts recovered from matrix samples spiked with 500 oocysts. The dashed lines indicate perfect correlations.

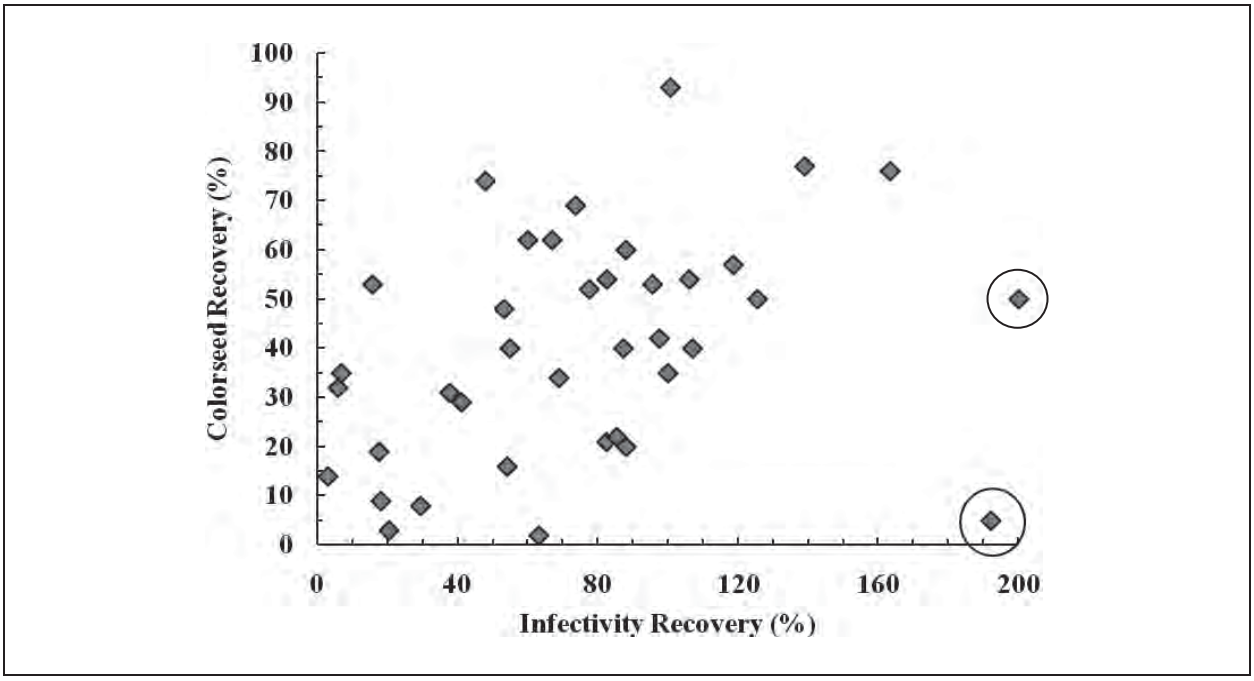


Figure 3.4 Comparison of matrix spike recovery efficiencies for all samples processed at both analytical laboratories. The circled data points are possible outliers due to unusually high values for infectivity recovery.

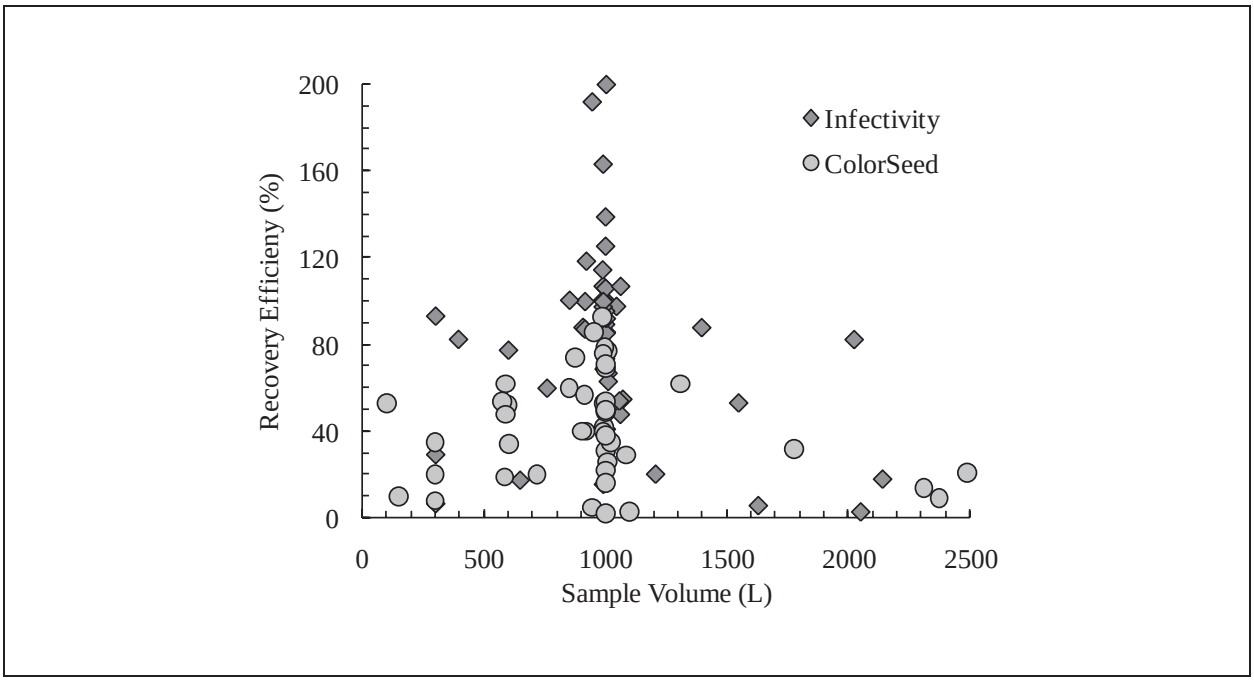


Figure 3.5 Correlation between filtered volume and recovery efficiency for matrix spike samples

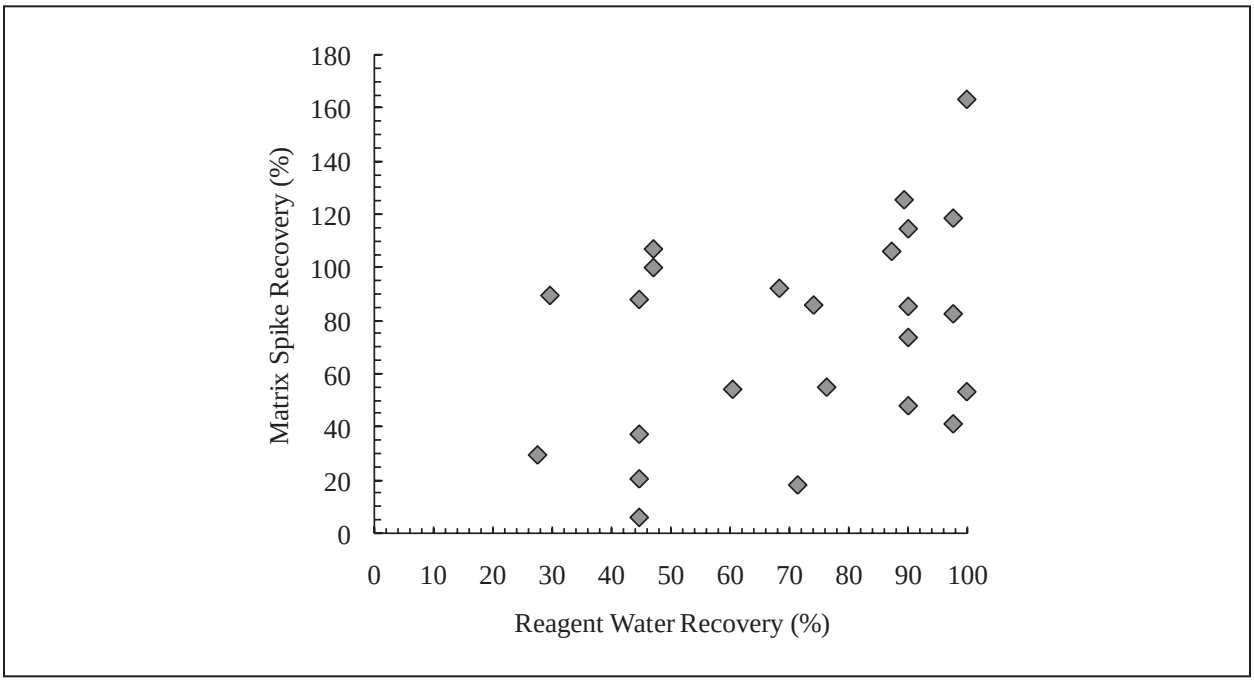


Figure 3.6 Comparison between infectivity recovery efficiencies for oocysts spiked into 10 L of reagent water and utility sample matrix spikes (average volume = 1,020 L)

of detecting infectious oocysts if they were present in the sample. Paired sample filters (1000 L of treated water each) from three participating utilities were spiked with infectious oocysts. Fresh *C. parvum* oocysts (Iowa isolate; Waterborne, Inc.) were sorted by flow cytometry (at WSLH) into tubes of 50 oocysts each, which were then spiked into sample filters (by CH Diagnostics). One filter from each utility was randomly chosen by CH Diagnostics and spiked with the flow cytometry enumerated infectious oocysts. The spiked and unspiked filters were then shipped to MWDSC for processing. The identity of the spiked filters was not revealed to the analysis laboratories until after the filters were processed.

Upon receipt at the MWDSC laboratory, the filters were eluted and the eluant concentrated by centrifugation and IMS. The magnetic beads were then removed from the sample with acidified Hank's Balanced Salt Solution/1% trypsin. The resulting sample was inoculated onto HCT-8 cell monolayers and incubated for 72 hours at 37°C. Infectivity of the oocysts was confirmed by inoculating six wells each with 50 and 500 flow cytometry enumerated oocysts. Infected monolayers were stained with anti-sporozoite antibody and FITC labeled secondary antibody to visualize the infectious foci.

The control oocysts had an average of 4–5 infectious foci per 50 oocysts inoculated onto the monolayer. This means that the dose spiked into the filters was equivalent to 4–5 infectious oocysts per filter. Infectious foci were detected on two of the three sample filters spiked with infectious oocysts and on the control filter (Table 3.8). No infectious foci were detected on the spiked filter from Utility 3. A high amount of algae was present in this sample, which may have interfered with the immunomagnetic separation of the oocysts from the sample. No infectious foci were detected on any of the unspiked filters.

This experiment confirmed that the elution and infectivity assay used for the detection of infectious oocysts from 1,000 L finished water samples was working as expected and should have detected infectious oocysts in utility samples, if they were present. The infectious dose spiked into the filters was equivalent to 4–5 infectious oocysts per filter and the recovery was two infectious foci for Utility 1 and 2. The results demonstrated that the procedure can detect less than five infectious oocysts in 1,000 L samples.

DETECTION OF NATURALLY OCCURING INFECTIOUS *CRYPTOSPORIDIUM* IN WASTEWATER SAMPLES

Although infectivity controls and matrix spikes with the *C. parvum* Iowa isolate demonstrated that the cell culture-IFA method was working consistently throughout Phase 2 of the project, additional experiments were performed to assess the method's ability to detect infection with naturally occurring oocysts in environmental samples. Waste water samples were used to maximize the likelihood of detecting infectious oocysts. Duplicate 10 L effluent grab samples were collected from a waste water treatment plant. The plant uses extended aeration activated sludge treatment and chlorine disinfection.

Sodium thiosulfate was added to collection containers to neutralize residual chlorine disinfectant. Samples were processed immediately upon returning to the AgriLife El Paso laboratory using the modified Method 1623-cell culture-IFA procedure described in Chapter 2. A laboratory blank (10 L of reagent water), cell culture infectivity positive (*C. parvum* Iowa oocysts) and negative controls (uninoculated cell monolayer) were included.

A single focus of infection was detected on one of the monolayers inoculated with an effluent sample, and DNA was extracted from the focus using the genotyping procedure described

Table 3.8
Infectivity assay of blind spikes

Filters		Volume filtered (L)	Total oocysts spiked	Infectious oocysts spiked*	No. infectious foci
Utility 1	a	1000	0	0	0
	b	1042	50	4.5	2
Utility 2	a	1020	0	0	0
	b	1020	50	4.5	2
Utility 3	a	1008	0	0	0
	b	1008	50	4.5	0
Control filter		10	50	4.5	3
Infectivity Assay Controls					
No. viable oocysts per monolayer			500	45	54.3†
			50	4.5	4.5‡
Gamma-irradiated oocysts			100		0
Unseeded wells			0		0

*Based on control infections.
†Mean, N=6.
‡Mean, N=6.

in Chapter 2. In addition, DNA was extracted from an area of the monolayer which showed no infection as a negative control, and also from a *C. parvum* positive control focus of infection. DNA samples were analyzed (in triplicate) using real-time PCR targeting the hsp70 gene and high-resolution melt curve analysis (Di Giovanni et al. 2009). The waste water effluent focus of infection sample tested positive, while the negative control sample tested negative. All other control samples generated the expected results. The effluent focus of infection appeared to be *C. hominis* or *C. meleagridis* since the hsp70 primers amplify only those species and *C. parvum* (LeChevallier et al. 2003), and the high resolution melt curves of the sample differed from the *C. parvum* control (Figure 3.7). DNA sequence analysis of amplicons identified the waste water infectious focus as *C. hominis* (99%–100% homologous to GenBank *C. hominis* sequences). Sequence analysis of the infectivity assay positive control generated the expected *C. parvum* identification.

An additional 5 L grab sample of waste water effluent was collected and processed. Two out of four purified subsamples produced infections with a single focus on one monolayer and eight foci on the second. Following microscopy, DNA was extracted from the foci of infection and amplified using hsp70 and GP60 primers. Based on DNA sequence analysis of both the GP60 and hsp70 amplicons, all these foci of infection were identified as the bovine genotype of *C. parvum*.

Therefore, these results clearly demonstrate that the complete method developed for this project (modified Method 1623-cell culture-IFA-genotyping) is capable of detecting infection with naturally occurring human-infectious oocysts and identifying the species of *Cryptosporidium* responsible for the infection.

DISCUSSION

There are a number of possible explanations for the lack of positive samples in this survey of infectious oocysts in finished water. First, and most likely, is that the results are accurate and none of the analyzed samples contained infectious oocysts because either there were no infectious oocysts in the utilities' source waters or those that were present were removed by the treatment

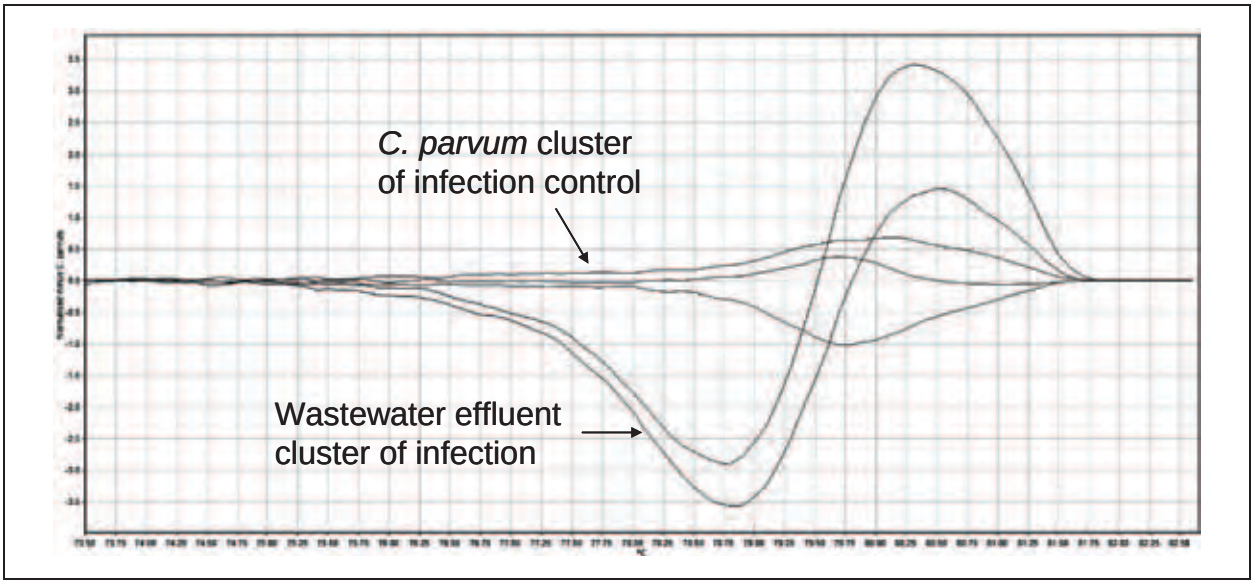


Figure 3.7 High resolution melt analysis of hsp70 real-time PCR amplicons from a wastewater effluent sample focus of infection and a *C. parvum* control focus of infection. All three PCR replicates of the *C. parvum* control amplified, whereas only two of three replicates of the effluent sample amplified.

processes. Since direct IFA enumeration of oocysts was not performed, it is not known whether any oocysts were present. Although a previous study detected infectious oocysts in finished water, it was a relatively rare event (Aboytes et al. 2004). Out of 1,690 samples from 82 utilities, 24 were positive (1.4%) and none of the repeat samples from these plants were positive. For the current study, we had difficulty recruiting utilities for the survey phase due to concern over the potential consequences of a positive result. Some utilities only agreed to participate after their state health departments provided an assurance that no action would be required other than follow-up samples and unofficial notification of the results. The utility recruitment and survey phases of the project were conducted primarily during the period covered by mandated *Cryptosporidium* monitoring for schedule 1 utilities under the LT2ESWTR (October 1, 2006–September 30, 2008). This raised the level of sensitivity to *Cryptosporidium* among utility managers. In addition, a media story concerning unreported detection of non-regulated contaminants in drinking water that received considerable public attention during this period added to utility sensitivity. Even for those utilities that did participate, there was a general sense of trepidation in getting involved in the project. This highlights the difficulty of conducting research in the public spotlight, particularly when there are potential regulatory, legal, public health, and public relations consequences attached to a positive result. Therefore, it is highly likely that the utilities that did participate were a self-selecting group that did not anticipate many, if any, positive samples. It is possible that a different group of utilities with a broader diversity of water quality characteristics, greater vulnerability of their source waters to *Cryptosporidium* contamination, and less rigorous treatment procedures and controls, might have produced some positive results. The previous study analyzed water from 82 treatment plants (Aboytes et al. 2004). Many of the plants that were positive in this earlier study are either no longer operating or have installed additional treatment and so were not suitable for the current study.

The remaining potential explanations for the lack of positive samples are method related, but can be generally discounted due to the various controls that were conducted. Positive controls

and routine matrix spikes indicated that the method was working, so the lack of positive samples was not due to false-negative results. Large volume samples (mean = 943 L) were filtered and processed using a modified version of Method 1623 followed by in-vitro cell culture. It is possible that infectious oocysts were lost somewhere in this process. However, all of the control experiments and matrix spikes indicated that infectious oocysts should have been detected if they were present. The average recovery efficiency for initial large volume spikes processed by Method 1623 alone was 71%. Although there were some low recoveries, the average recovery efficiency for ColorSeed oocyst matrix spikes for all of the utilities was 41%. Finally, the recovery efficiencies for matrix spikes using freshly shed oocysts and processed through the entire procedure, including cell culture were 3–200% with a mean of 80% (N = 51). So, although recovery efficiencies were not all 100% (as expected), these control results indicate that positive samples would have been detected.

The IFA cell culture detection method was tested for the ability to detect infectious oocysts in very low numbers. Cell monolayers were infected with three flow cytometry enumerated oocysts per well and both MWDSC and AgriLife El Paso laboratories processed 80 wells each. Table 2.10 shows that 97% of the infectious oocysts present in the inoculum were detected by IFA. This indicates that if infectious oocysts had been present in the sample, the IFA cell culture detection method would have detected them.

The operational definition of infection adopted for this project required detecting at least three fluorescing objects of the correct size, morphology, and color on the cell monolayer. Therefore, an inoculated monolayer containing only a single green fluorescing object of the correct size was considered negative for infection. Fifty-three of the 370 monolayers processed for the finished water survey contained green fluorescing objects on the monolayer but did not meet the project's definition of infection. The objects were not clustered closely together nor were they the correct size of *Cryptosporidium* life stages.

It is possible that oocysts were present but they belonged to species or genotypes that are unable to infect HCT-8 cells. We have demonstrated in this and other studies (Rochelle et al. 2002) that all three of the major human-infectious *Cryptosporidium* species (i.e., *C. parvum*, *C. hominis*, and *C. meleagridis*) can infect HCT-8 cells and their infectious foci are detected using the IFA assay. It has previously been reported that *C. andersoni* infects HCT-8 cells (Hijjawi et al. 2002; Wu et al. 2009) but this finding was not replicated. This may be due to lack of infection or inability of the Waterborne SporoGlo antibody to stain *C. andersoni* infectious foci. Also, no infection was detected with *C. muris*. Consequently, the diversity of *Cryptosporidium* species and genotypes that will infect HCT-8 cells and be detected using the IFA procedure used in this study is currently unknown. Nevertheless, from a public health perspective, the current assay detects the most important human-infectious species.

The positive samples detected in the Aboytes study translated to an annual cryptosporidiosis risk of 52 infections per 10,000 people, based on Equation 3.1. The lack of positives in the current study translates to an annual risk of less than one infection per 10,000 people using the same equation but substituting the larger volume of water analyzed (349,053 L versus 169,000 L). In implementing the Surface Water Treatment Rule in 1989, the USEPA determined that an acceptable annual risk of infection (the chance of one person being infected during one year) of 1/10,000 should be the goal of water treatment plants. In calculating this number, the recovery efficiency of the method, the concentration of the oocysts in water, and the infection index of the organism (the ability of the oocyst to cause an infection if ingested) must be considered. A frequent assumption for these calculations is that the average person ingests 1.2 L of unboiled tap water per day, but changing consumer habits and the increasing popularity of bottled water add unknown variability to this assumption. A

more accurate estimate of average water consumption is 0.93 L/day (USEPA 2005), although this is probably high since it was based on community water consumers only, which excluded those individuals that reported never drinking tap water. Estimates for daily risk of *Cryptosporidium* infection are typically in the range 1.5×10^{-5}–3.8×10^{-4}. However, most of these estimates result in annual disease burdens that are orders of magnitude higher than the reported incidence of cryptosporidiosis cases from all sources in the U.S. In 2007, the Centers for Disease Control reported 11,170 cases of cryptosporidiosis from all sources nationwide with an annual average of 4,261 cases for the 10 years covering 1997–2007. The average annual incidence in the U.K. was 5.9–11.6/100,000 for a similar period. Since risk assessments are sensitive to the assumptions and values underlying the calculations, a variety of model scenarios were evaluated to determine the number of positive samples that would have been necessary in the current study to exceed a 1 in 10,000 risk (Table 3.9). These calculations were based on a total analyzed volume of 349,053 L, exposure to drinking water for 365 days per year, various volumes for consumption of unboiled drinking water, and various *Cryptosporidium* infection indices. In the worst case scenario from a public health perspective (large volume of water consumed, high infection index, and low method recovery efficiency based on ColorSeed spikes) the minimum number of positive samples that were needed in this study to exceed a 1 in 10,000 risk was two. At the other end of the public health scale (low water consumption volume, low infection index, and higher recovery efficiency), approximately 10-fold more positives would have been necessary. Fifty samples should have been positive to obtain the annual risk of 52 infections per 10,000 people calculated by Aboytes et al. (2004).

The results of this risk assessment model cannot be applied generally to the populations served by the 14 utilities because the calculations were based on the total sample volume (349,053 L) and some plants were sampled more frequently, over a longer period of time, than others. Nevertheless, the results from these 14 plants indicate the occurrence of infectious *Cryptosporidium* in conventionally treated drinking water in some areas of the U.S., produced by correctly operating treatment plants, may be lower than previously thought.

There can be no doubting the significance of waterborne cryptosporidiosis as a serious public health concern. The Milwaukee outbreak in 1993 affected an estimated 400,000 people (Mackenzie et al. 1994) and outbreaks continue despite greater awareness within the water industry and increased regulation. Nevertheless, many factors combine to introduce a high level of uncertainty into determining the actual contribution of drinking water to cryptosporidiosis in the community. These include:

- Widely varying oocyst occurrence data in source and finished waters
- Detection methods with variable recovery efficiencies
- Various measures of oocyst viability
- Non-standardized infectivity assessment methods
- Relatively little information on the infectivity of oocysts in source and finished waters
- Differing risk assessment models and underlying assumptions
- Varying susceptibility to infection within different sub-populations
- The potential role of protective immunity resulting from low-level endemic exposure
- Varying infectious doses for different strains of *Cryptosporidium* spp.
- Inadequate detection and reporting of endemic cases and outbreaks
- The contribution of other common routes of infection (e.g., food, swimming, person-to-person)
- The popularity of international travel to countries with less stringent regulations and treatment practices

Table 3.9
Risk of waterborne cryptosporidiosis

Water consumption (L/day)*	Recovery efficiency (%)†	Infection index‡	No. of positives to exceed 1/10,000 risk
0.27	41.5	0.0053	29
0.27	79.5	0.018	16
0.27	70.5	0.018	14
0.27	41.5	0.018	9
0.27	79.5	0.028	11
0.27	70.5	0.028	9
0.27	41.5	0.028	6
0.6	41.5	0.0053	13
0.6	79.5	0.018	8
0.6	70.5	0.018	7
0.6	41.5	0.018	4
0.6	79.5	0.028	5
0.6	70.5	0.028	5
0.6	41.5	0.028	3
1.2	41.5	0.0053	7
1.2	79.5	0.018	4
1.2	70.5	0.018	4
1.2	41.5	0.018	2
1.2	79.5	0.028	3
1.2	70.5	0.028	3
1.2	41.5	0.028	2

*Water consumption values are the widely used 1.2 L/day, half of this value (0.6 L/day), and the median value from a study of sporadic cryptosporidiosis cases (0.27 L/day; Goh et al. 2005).
†Recovery efficiencies were based on recovery of ColorSeed oocysts using the modified Method 1623 (41.5%), recovery of Iowa oocysts in the initial large volume matrix spikes (70.5% from Table 3.1), and the mean infectivity recovery for the entire method by both analysis laboratories (79.5% from Table 3.3).
‡Infection indices for an unknown strain in a population (0.028), a mix of the Iowa, TAMU, and UCP isolates (0.018), and the Iowa isolate only (0.0053; Messner et al. 2001).

Haas et al. (1996) used human dose response data to calculate a theoretical acceptable daily oocyst intake of 6.5×10^{-5}. Based on water consumption of 1.5 L/day/person, the theoretical maximum acceptable concentration was 4.4×10^{-2} oocysts/1,000 L, which is below the detection limit of currently used monitoring methods. Perz et al. (1998) used a risk assessment model to determine the potential role of tap water in the transmission of endemic cryptosporidiosis in New York City. Based on a concentration in finished water of 1 oocyst/1,000 L, it was estimated that tap water was responsible for an annual disease incidence within the city of 6,000. A more recent multiplicative model estimated the daily infection risk for the general immune-competent population in New York City as 3–10 cases per 100,000 people (Makri et al. 2004).

A case-control study of sporadic cryptosporidiosis among 282 immunocompetent individuals in seven states reported that the most significant risk factors for becoming infected were international travel, contact with cattle, contact with young children suffering from diarrhea, and swimming in freshwater (Roy et al. 2004). Consumption of well water within Minnesota was a significant risk factor but not in the other six states in the study. However, for the whole study,

drinking water was not associated with *Cryptosporidium* infection. Similarly, a case-control study of immunocompetent individuals in the San Francisco Bay Area (N = 26 cases and 62 controls) found no significant association between cryptosporidiosis and consumption of tap water (Khalakdina et al. 2003). The major risk factor for cryptosporidiosis amongst this population was travel to another country. The authors concluded that drinking water is not an independent risk factor for cryptosporidiosis among the immunocompetent population. In contrast, a case-control study with 49 subjects concluded that up to 85% of endemic cryptosporidiosis cases in AIDS patients in San Francisco could be attributed to consuming tap water (Aragon et al. 2003) although no waterborne outbreaks of cryptosporidiosis had ever been detected in the city up to the time of the study. This led the authors to recommend that AIDS patients, particularly those with reduced immune function, should avoid tap water. Current national guidelines in the U.S. also recommend that HIV-infected individuals avoid consuming unboiled tap water.

There are approximately 5,000 reported cases of cryptosporidiosis each year in the U.S. (Table 3.10). However, many cases of cryptosporidiosis in the community are not reported to national surveillance programs. This under-reporting may increase the actual incidence by 7.4-fold or higher (Adak et al. 2002), which would increase the annual incidence in the U.S. to 35,291 cases. However, under-reporting of cryptosporidiosis may be much higher than 10-fold, in which case the national cryptosporidiosis burden could be substantially higher. The previous study on the occurrence of infectious *Cryptosporidium* in finished drinking water reported an annual risk of 52 infections per 10,000 people, which translates to a U.S. national risk of approximately 1.6 million cases per year.

Drinking water regulations introduced in England and Wales in 1999 (DWI, 1999) significantly reduced the incidence of cryptosporidiosis during the first six months of each year but there was no significant change during the second half of the year (Lake et al. 2007). The authors estimated that there were approximately 6,770 fewer cases of cryptosporidiosis per year as a result of the new regulations (number based on reported incidence and under-reporting multiplier). An annual incidence of approximately 22 cryptosporidiosis cases per 100,000 people declined to <10 cases per 100,000 people, coincident with installation of membrane filtration at two treatment plants and a nationwide foot and mouth disease outbreak that led to widespread culling of livestock, reduced travel to the countryside, and restricted livestock movement (Goh et al. 2005). The authors concluded that drinking cold unboiled municipal tap water was a leading independent risk factor for sporadic cryptosporidiosis. However, considering the significant decrease in cases observed in the control community that did not have membrane filtration installed, it is difficult to determine the true contribution of membrane filtration to the reduction in disease incidence and consequently, the actual contribution of drinking tap water to the overall disease burden was uncertain.

Until October 2008, the UK drinking water regulations included the most intensive *Cryptosporidium* monitoring program ever undertaken. The regulation required continuous monitoring of *Cryptosporidium* oocysts in finished drinking water for at least 23 hours per day at a flow rate of at least 40 L per hour (DWI, 1999). Although the majority of samples analyzed during this decade-long monitoring program were negative, *Cryptosporidium* oocysts were occasionally detected in finished drinking water. During the period 2000–2002, a total of 97,999 samples were analyzed (total volume = 115,303,050 L), 5.5% were positive, and the average oocyst concentration was 0.0002 oocysts/L (Smeets et al. 2007). In the earlier years of the monitoring program, oocysts were detected at least once in the finished water from many plants. For example, in 2002, 1.9% of samples were positive (N = 47,049) but oocysts were detected at least once from 68% of sample sites (DWI, 2002). Similarly, in 2003, 1.1% of samples were positive (N=57,529) with

Table 3.10
Incidence of cryptosporidiosis

	Reported cases of cryptosporidiosis	
Year	United States*	England and Wales†
2007	10,080	
2006	5,400	
2005	5,659	
2004	3,577	3,514
2003	3,506	5,437
2002	3,016	2,898
2001	3,785	3,386
2000	3,128	5,367
Mean	4,769	4,120

*Data from Centers for Disease Control database of notifiable diseases.
†Data from Lake et al. 2007.

54% of sample locations reporting at least one detection. So clearly, the public is being exposed to low levels of *Cryptosporidium* oocysts in finished drinking water. Nevertheless, the results of this extensive monitoring program allowed the DWI to conclude that treated drinking water is not a major source of exposure of the population to *Cryptosporidium* oocysts. In 2008, a total of 50,569 samples were analyzed (total volume = 46,523,480 L) from 204 plants but none of them exceeded the treatment standard of <1 oocyst/L (DWI, 2008).

There are many studies that suggest sources other than drinking water may commonly transmit *Cryptosporidium*. Food and other modes of parasite transmission may be at least as important as drinking water and may be more likely to transmit higher dose exposures (Frost et al. 2005). In fact, some studies indicate that low level endemic exposure to oocysts in drinking water confers protective immunity that protects individuals during outbreaks (Frost et al. 2005; Chappell et al. 1999). This has led some authors to suggest that "the emergence of cryptosporidiosis as a serious epidemic disease in Western countries resulted largely from reduced levels of low-dose exposure and protective immunity. Protective immunity likely declined after improvements in sanitation and drinking-water treatment" (Frost et al. 2005).

Based on the UK's finished water monitoring results of 0.0002 oocysts/L, we could have expected 70 oocysts in the 349,053 L that were analyzed for this project. Adjusting this value to account for Method 1623 recovery efficiencies ranging from 44–90%, the expected number of oocysts is reduced to 31–63. Since approximately 10% of oocysts in a freshly shed sample can initiate an infection (see Chapter 2), only 3–6 of these oocysts were likely to be infectious. Consequently, even though 349,053 L of water were analyzed, the likelihood of "hitting" the one or two samples that might have contained infectious oocysts was low.)

Detection of *Cryptosporidium* oocysts in finished water during the UK's regulatory monitoring program and detection of infectious oocysts in finished water (Aboytes et al. 2004) demonstrates that conventional treatment with granular media filters does not remove 100% of oocysts. So oocysts will infrequently breakthrough the treatment barrier into treated water. Therefore, it is critically important to determine whether these oocysts are genotypes that are infectious to humans. Since monitoring 349,053 L of treated water from 14 plants was insufficient to assess the prevalence of infectious oocysts, a much larger volume of water from many more treatment plants should be analyzed. This could be accomplished by incorporating cell culture-based infectivity testing into the second round of monitoring under the LT2ESWTR.

CHAPTER 4
SUMMARY AND CONCLUSIONS

Cryptosporidium in drinking water continues to be a public health concern. Almost two decades after the Milwaukee incident, outbreaks of cryptosporidiosis still occur, linked to both drinking water and recreational water. Research studies have reported high levels of oocyst occurrence in untreated and finished drinking water although monitoring programs typically demonstrate lower occurrence values. The proportion of positive source water samples in the Information Collection Rule monitoring was 6.8% with a mean concentration of 0.067 oocysts/L (N = 5,838; Messner and Wolpert 2003). The first round of monitoring under the LT2ESWTR will probably result in most utilities being placed in Bin 1 (<0.075 oocysts/L). However, given the uncertainty surrounding the overall contribution of drinking water to the national cryptosporidiosis disease burden, efforts have been made to estimate the risk to public health from *Cryptosporidium* in water.

The currently approved method for detecting *Cryptosporidium* oocysts in untreated surface water use filtration through 1 µm porosity capsule filters (or other filtration options), elution and centrifugation, oocyst purification by immunomagnetic separation, and enumeration by immunofluorescence microscopy (Method 1622 and 1623; USEPA, 2005). This method only provides presence/absence detection of oocysts. The absence of sporozoites within the oocyst (determined by DAPI staining and/or DIC microscopy) suggests that the oocyst is not infectious but the presence of sporozoites does not mean that the oocyst is infectious to humans. An intact oocyst may not be *C. parvum* or *C. hominis* or the oocyst may be sufficiently damaged that it will not cause infection in humans. The detection of non-infectious oocysts or oocysts belonging to a species that is not infectious for humans could cause unwarranted concern for a contaminant that might not be a significant public health risk. Consequently, accurate risk assessments need data on the infectivity of oocysts in water.

A previous study reported relatively high occurrence of infectious *Cryptosporidium* in finished drinking water (1.4% of samples) concluding that conventional treatment plants were not achieving the USEPA's 1 in 10,000 annual risk of infection goal; the annual risk range was 9 – 119 infections per 10,000 people (Aboytes et al. 2004). The Aboytes study used HCT-8 cell culture with cells grown in 96-well plates, incubated for 72 hours in a 5% CO_2 atmosphere. Infections were detected by PCR amplifying a 346 bp amplicon from the hsp70 gene. Genotyping identified one isolate as *C. hominis* and 23 isolates as *C. parvum*, with sequences distinct from the laboratory strain demonstrating that the positive samples were not due to laboratory contamination.

The primary objective of the current study was to determine whether the findings of this earlier study could be replicated. If so, the data would confirm that many people in the U.S. are being served water that does not meet the 1 in 10,000 annual risk goal. The two studies did not use the same method to detect infectious oocysts, and arguments can be made that this was either a weakness or strength of the current study. To allow a direct evaluation of reproducibility with the earlier study, this project should have used PCR targeting hsp70 DNA to detect infections. However, in side-by-side comparisons of three infectivity detection methods, PCR targeting hsp70 DNA had the highest rate of false-positives for inactivated oocysts and oocysts remaining on monolayers from mock infection controls (see Chapter 2). Although the Aboytes et al. study may have included some false-positives for the presence of infectious oocysts, it did unequivocally indicate that *C. parvum* and *C. hominis* oocysts were present in finished drinking water samples.

Since there was no standardized method for *Cryptosporidium* cell culture assays at the start of this project, three published assays were compared for their ability to detect infection in HCT-8 cell monolayers. The three infectivity detection methods were PCR targeting hsp70 DNA (Di Giovanni et al. 1999), RT-PCR targeting hsp70 mRNA (Rochelle et al. 2002), and an immunofluorescence microscopy assay (IFA; Slifko et al. 1997). The methods were evaluated based on their sensitivity, reproducibility, robustness, rates of false-positives, and ease of use. Based on both quantitative and qualitative comparisons, IFA was selected as the most appropriate infectivity detection method for assessing the occurrence of infectious *Cryptosporidium* in finished water samples. It consistently detected infections with low oocyst numbers (≤3 oocysts), generated few false-positives (all of which could be discounted by an experienced microscopist), was reproducible, and relatively simple to perform. Using a different infectivity detection method than the original Aboytes et al. study provides the advantage of independent corroboration with an entirely different technique (if positives were detected).

Previous studies have demonstrated the equivalency of cell culture assays to animal models and the methods evaluation phase of this project (Chapter 2) demonstrated that cell culture is a practical and sensitive method for assessing the infectivity of *Cryptosporidium* in finished drinking water. HCT-8 cell culture with infections detected by RT-PCR targeting hsp70 mRNA was equivalent to infection in CD-1 mice with respect to sensitivity, reproducibility, variability, 50% infectious dose for multiple isolates of *C. parvum*, and measuring UV disinfection (Rochelle et al. 2002, 2004). Based on 31 dose-response trials there was a significant correlation between HCT-8 cell culture combined with IFA detection and mouse models and cell culture was equivalent to mice for measuring UV and chlorine dioxide disinfection (Slifko et al. 2002). In addition, HCT-8 cell culture combined with IFA accurately predicted the number of infectious oocysts in blind samples (Bukhari et al. 2007). Cell culture has the added benefit of supporting infection of *C. hominis* oocysts (Rochelle et al. 2002; Johnson et al. 2005), which will not replicate in standard mouse models. Cell culture-based methods have been used to evaluate infectivity of different species and strains of *Cryptosporidium* spp. (Di Giovanni and LeChevallier 2005; Johnson et al. 2005; Rochelle et al. 2002), UV disinfection of multiple species and strains (Entrala et al. 2007; Mofidi et al. 2001; Johnson et al. 2005; Rochelle et al. 2004), various chemical disinfectants (Keegan et al. 2003; Joachim et al. 2003), drug efficacy (MacDonald et al. 2002; Shahiduzzaman et al. 2009), and oocyst survival in natural waters (Ives et al. 2007; Johnson et al. 2008).

However, due to the variability inherent in using complex living systems to measure the activity of another organism, both cell culture and mouse models should be limited to discerning infectivity differences of >0.3 log (Rochelle et al. 2002). Sources of variability in cell culture-based infectivity assays include: oocyst propagation and handling procedures; asynchronous susceptibility to infection of individual cells within a cell monolayer; and differences in host cell handling procedures.

In the current project, we demonstrated that a standardized cell culture assay could be applied to environmentally-relevant low numbers of oocysts recovered from large volumes of finished water using a modified version of USEPA Method 1623. The standardized cell culture method involved incubating inoculated HCT-8 cells in 8-well chamber slides at 37°C for 72 hours, fixing monolayers in methanol, staining with an anti-sporozoite antibody and FITC-labeled secondary antibody, and enumeration of infections by epifluorescence microscopy. A detailed procedure is provided in Appendix B.

This project compared three methods for detecting infection in HCT-8 cells and selected and standardized the most appropriate method for an occurrence study. However, there is still

scope for improving the cell culture assay. For example, oocyst infectivity is typically 5–15%, based on the number of infectious foci per inoculum oocyst (5–15 foci per 100 oocysts inoculated onto the cell monolayer). The reasons it is not 100% (one infectious focus per each inoculated oocyst) or 400% (four infectious foci from each oocyst containing four sporozoites) may include:

- Susceptibility of the host cells to infection.
- Asynchronous host cell replication cycles.
- Physiological status of the oocyst.
- Only some oocysts in a fresh population are capable of initiating an infection, possibly due to asynchrony of the oocyst life cycle.
- Not all sporozoites within an oocyst may successfully infect.
- Foci from independent sporozoites or oocysts may overlap.
- Laboratory protocols may not achieve 100% infectivity.

However, none of these factors should preclude the use of a cell culture assay for assessing the prevalence of infectious *Cryptosporidium* in finished drinking water. Supplementing cell culture medium with various vitamins and other compounds increased infectivity of *C. parvum* (Upton 1997). Also, 65% of nutrient depleted HCT-8 cells were infected compared to only 15% of control cells grown in normal medium (Perez Cordon et al. 2007). Adding 1 mM $MgCl_2$ and $CaCl_2$ also increased the proportion of host cells that were parasitized. Consequently, there are a variety of approaches that can be considered for increasing the sensitivity of cell culture assays. However, since physiological status of individual oocysts and oocyst population biology have a role in the level of infectivity, it is unlikely that manipulating host cell growth conditions will result in consistent 100% infectivity.

We demonstrated that the anti-sporozoite antibody bound to and allowed detection of life-cycle stages produced by *C. parvum*, *C. hominis*, and *C. meleagridis* oocysts. Therefore, if infectious oocysts of these three species were present in finished water samples, their resulting infectious foci would have been detected. So from a public health perspective, the method is sensitive to the most important species. However, there is little information regarding the infectivity of other species in HCT-8 cells. Also, the infectious stages of other species may not be detected by the antibody, even if they infect the monolayer and produce foci. Therefore, more research is necessary to determine the diversity of species and genotypes that infect HCT-8 cells and the ability of the detection method (e.g., IFA using a variety of antibodies) to detect these infections.

A total finished water volume of 349,053 L in 370 samples from 14 treatment plants was analyzed by cell culture and no infectious oocysts were detected (see Chapter 3). All of the controls, matrix spikes, and blind spiked samples indicated that the method was working as expected, so the absence of positive results cannot be attributed to failures of the method. The lack of positives in the current study translates to an annual risk infection of <1 in 10,000 for the populations served by these 14 treatment plants using a previously described risk estimate calculation (Aboytes et al. 2004) but substituting the larger volume of water analyzed (349,053 L versus 169,000 L). Applying the risk calculation to the individual utility with the largest volume of water analyzed (46,606 L), the risk was <8 in 10,000. According to the USEPA's SWTR, the goal of conventional water treatment plants should be a maximum annual risk of *Cryptosporidium* infection of 1 in 10,000. The results from these 14 plants indicated that the occurrence of infectious *Cryptosporidium* in conventionally treated drinking water in some areas of the U.S., produced by correctly operating treatment plants, was low and drinking water meets this risk goal. However, it

is difficult to determine the number of utilities and volume of water that must be sampled to provide a representative cross-section of drinking water utilities so that project results can be extrapolated to the broader community. The combined capacity of the plants sampled for this project was approximately 1.9 billion gallons per day, serving almost 9 million people.

Most of the utilities that participated in this study will probably be classified in Bin 1 under the LT2ESWTR (<0.075 oocysts/L). Two utilities may be classified in Bin 2, based on historical source water oocyst levels. However, monitoring data is usually collected over long periods of time and averaged, so an individual sample collected during a storm event could contain significantly more oocysts than the average. With a source water oocyst concentration of <0.075 oocysts/L, and assuming 2-log removal by conventional treatment, the maximum oocyst concentration in finished water would be 0.75 oocysts in a 1,000 L sample. Consequently, the likelihood of an infectious oocyst occurring in any single 1,000 L sample was low, although the cell culture-IFA method will detect a single infectious oocyst if it is recovered from the sample and successfully introduced to the cell monolayer.

The inability of the current project to replicate the findings of the Aboytes et al. (2004) study adds further uncertainty to determining the actual risk of cryptosporidiosis from drinking water and highlights the difficulty in calculating a national average risk using data from a few select utilities. If all samples are negative (as in this project) the calculation may underestimate the national risk. Conversely, focusing just on utilities with high occurrence in source waters (and consequently more likelihood of detecting infectious oocysts in finished water) may overestimate the national average risk. Estimating a nationwide risk of infection may not be practical or meaningful considering the following factors:

- Most utilities are likely to be in Bin 1 and the majority will not have detectable infectious oocysts in finished drinking water.
- There is considerable uncertainty surrounding infectious doses estimates.
- Variability in occurrence data.
- Variability in treatment practices.
- Varying sensitivity to infection of different human sub-populations.

This project highlighted the difficulty in applying a non-compliance microbiological method when the results could have adverse legal, operational, public health, and public relations consequences for participating utilities. Utilities were reluctant to participate because of concerns over the possible consequences of detecting infectious oocysts in their finished drinking water. Since a broader range of utilities needs to be surveyed, a possible solution to the lack of voluntary participation is for regulatory agencies to mandate infectivity analyses on finished water during the second round of *Cryptosporidium* monitoring under the LT2ESWTR. While this would not be practical for all utilities, a subset of large and mid-size utilities could be monitored on a relatively frequent basis. The cell culture method is sufficiently developed and standardized that the laboratory capacity could be readily built within the regulatory timeframe. Options for implementing cell culture-based infectivity monitoring include (in decreasing order of complexity for utilities):

- On-site cell culture facilities at utility laboratories.
- Purchasing ready to use cell monolayers from a commercial supplier and then performing oocyst recovery and infectivity assay procedures in-house.
- Shipping recovered oocysts to a centralized cell culture testing facility.

Some of the Method 1623-approved contract laboratories have already installed cell culture facilities and implemented contract infectivity testing. Although coordination between the logistics of cell culture (e.g., cell split schedules) and receipt of oocyst samples has been seen as problematic, HCT-8 monolayers up to three weeks old were as sensitive to infections as fresh (≤48 hours) monolayers (Sifuentes and Di Giovanni 2007). Therefore, the ability to use aged monolayers should make cell culture feasible for a wider range of water quality and contract laboratories.

If this or a similar project is repeated and relies on voluntary participation by utilities, federal and state regulatory agencies and public health departments should be part of the project planning and design from the outset to encourage utility participation. In addition, utilities must be assured by their respective regulatory authorities that they will not be legally liable if infectious oocysts are detected, although mitigation measures would be expected. Also, analysis laboratories should be blinded to the identification of the utilities so that there is no possibility of linking infectivity results to any individual utility.

CONCLUSIONS

1. Infectious oocysts were not detected in 349,053 L of finished drinking water from the 14 treatment plants participating in the study.
2. The annual risk of infection for the populations served by these treatment plants, based on zero detects and the total volume of water analyzed, was <1 in 10,000.
3. Cell culture-based detection assays are sufficiently mature and standardized to be used for assessing the infectivity of *C. parvum* and *C. hominis* oocysts in finished drinking water.
4. The cell culture assay detected infection with *C. parvum, C. hominis,* and *C. meleagridis* but not *C. andersoni* or *C. muris*.
5. Oocysts can be recovered from large volumes (≥1,000 L) of finished water using a minor modification of USEPA Method 1623 and applied to cell monolayers to assess their infectivity.
6. Comparing three infectivity detection assays demonstrated the superiority of IFA over PCR and RT-PCR, based on qualitative and quantitative measures of performance.
7. Genotyping can be incorporated into non-molecular methods of infectivity detection methods such as cell culture-IFA, so that infectious oocysts can be identified to the species and sub-species level.
8. The entire method consisting of oocyst recovery by a modified Method 1623, inoculating HCT-8 cells, detecting infection by IFA, and genotyping, can be applied to naturally occurring oocysts in environmental water samples.)

RECOMMENDATIONS

1. Implement monitoring for infectious *Cryptosporidium* oocysts in finished water using a standardized cell culture assay. These assays may be carried out using in-house facilities or contract laboratories.
2. Conduct follow-up studies that include state public health professionals and federal regulators as part of the project team. This expanded team may help to reduce the reluctance of utilities to participate.

3. Focus future surveys on Bin 2 or higher utilities rather than attempting to capture a national average risk of infection. Bin 2 and higher utilities represent an increased risk of infection compared to the majority of plants, which will be classified as Bin 1. Surveys could include intensive sampling of a few plants over an extended period.
4. Optimize the *Cryptosporidium* cell culture method, to increase proportional infectivity, which will increase the likelihood of detecting infection with a single oocyst.
5. Assess the range of *Cryptosporidium* species and genotypes that can infect HCT-8 cells and the specificity of the anti-sporozoite antibody to infectious stages of species other than *C. parvum, C. hominis,* and *C. meleagridis*.

APPENDIX A
SAMPLE COLLECTION MANUAL

Detection of Infectious *Cryptosporidium* in Filtered Drinking Water

Sample Collection Manual for 1,000 L of Finished (Treated) Water using USEPA Method 1623

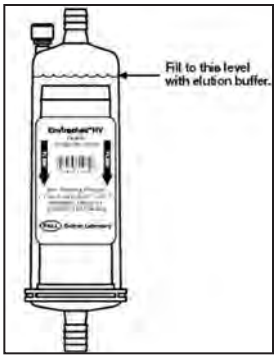
OVERVIEW OF PROCESS

This process requires the collection of large volume finished water samples from drinking water utilities. The sample collection method is essentially the same as USEPA Method 1623, which was developed for smaller volumes of untreated source water (USEPA, 2005. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/IFA; available as a downloadable file at www.epa.gov/nerlcwww/1623de05.pdf).

Treatment plant effluent water samples (1,000 L) will be filtered using Envirochek HV filter capsules. Chlorine residual will be neutralized by in-line addition of sodium thiosulfate. The filter capsules will be shipped on ice and eluted at the laboratory. The presence of infectious oocysts will be determined by an in-vitro cell culture assay.

EQUIPMENT AND SUPPLIES

Envirochek HV sampling capsules, Pall Gelman Laboratory, Ann Arbor, MI; product no. 12098.



Blue-White flow control meter, Harrington Industrial Plastics (www.harringtonplastics.com); product no. F-45375LEA-8.

Proportioning injector, DEMA Engineering Co. (www.demaeng.com); model 204B-½."

Flow totalizing meter, Daniel J. Jerman Co. (www.watermeters.com; model DLJSJ50-M3 or equivalent, must measure flow rate from 3–100 gpm).

Tygon reinforced tubing, ½" ID x ¾" OD, Ryan Herco (www.ryanherco.com); product no. 0030-138.

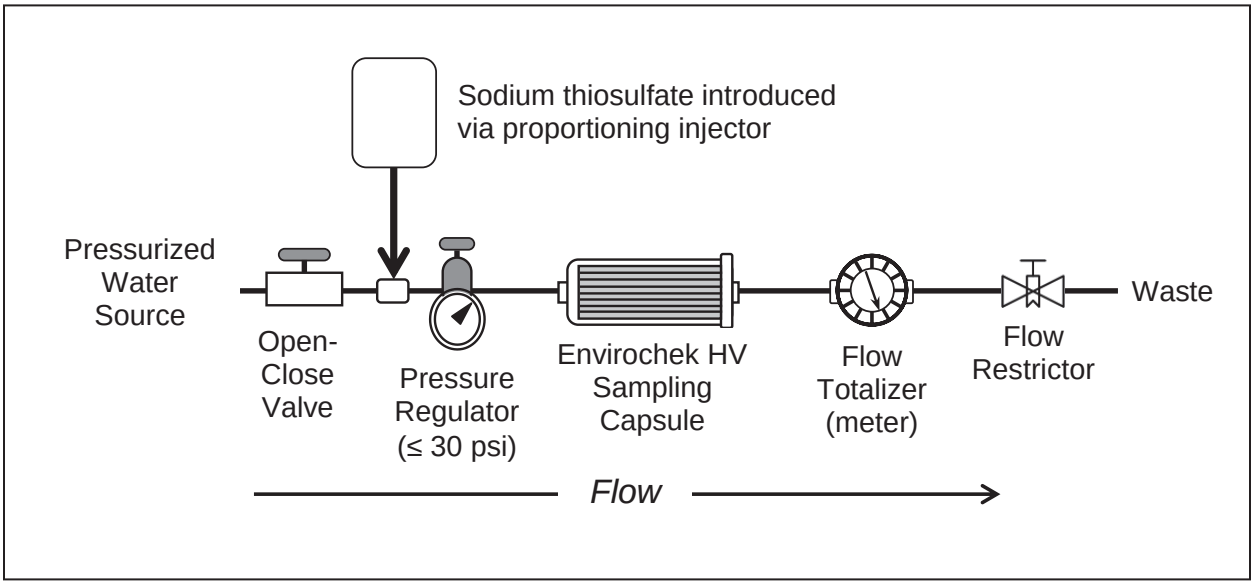
Sodium thiosulfate solution, 15 L of a 2% (w/v) solution:

1. Using a 2 L beaker, add 300 g sodium thiosulfate pentahydrate (Sigma no. S8503) to approximately 1 L reagent water.
2. Stir until dissolved.
3. Transfer the contents of the beaker to a 20 L autoclavable carboy (VWR # 36494-092 or equivalent) and QS to 15 L with reagent water.
4. Stir to completely mix.
5. Autoclave contents for 30 minutes at 121°C prior to use.

If the utility cannot provide filters, Envirocheck HV filters will be shipped to the utility. If supplied by the utility, the recovery efficiency for each lot of filters will be needed. Recovery efficiencies are determined by filtering a representative volume of finished water, spiked with 100 flow-cytometry enumerated, gamma irradiated (inactivated) oocysts, and recovering the oocysts using Method 1623. The recovery efficiency is the number of oocysts recovered expressed as a percentage of the spike number.

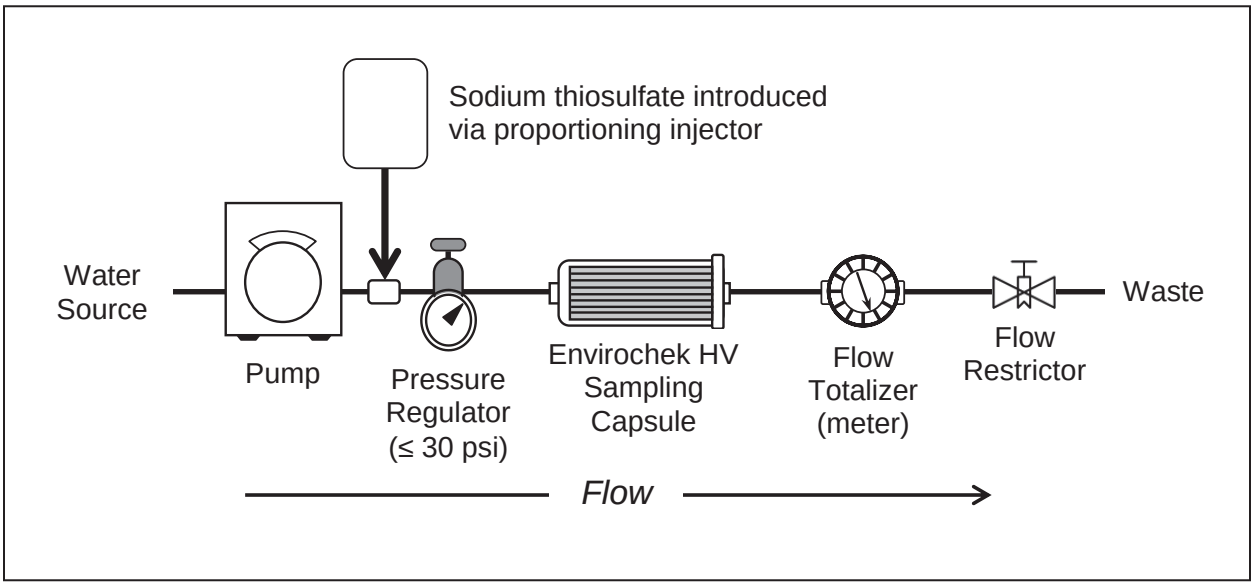
The sampling apparatus consists of tubing, proportioning injector (for sodium thiosulfate neutralization of chlorine residual), flow control valve, and flow meter. A peristaltic pump may be necessary if the pressure from the sampling tap has an insufficient flow rate. This apparatus will be supplied to the utility if the utility does not have or cannot acquire the equipment.

EQUIPMENT SET UP FOR A PRESSURIZED SOURCE OF WATER



The pressure regulator and flow restrictor may not be necessary, depending on the particular characteristics of the sample tap and water flow rate.

EQUIPMENT SET UP FOR A NON-PRESSURIZED SOURCE OF WATER OR INSUFFICIENT FLOW-RATE



SAMPLE COLLECTION, STORAGE, AND SHIPMENT

1. Connect the inlet end of the sampling hose to a pressurized tap.

   Flow rates for filtration can range from 0.7 L/min (total run time approx. 24 hours) to 4 L/min (total run time approx. 4.2 hours) to facilitate the schedules of the utilities and their employees. Do not exceed 30 psi or 4 L/min or the filter integrity may be compromised (use a pressure regulator upstream of the filter if necessary). Adjust the flow rate as necessary by opening or closing the flow meter valve.

   1,000 L will be filtered for regular samples and 900 L will be filtered for matrix spike samples.

Sample tap

Flow meter adjustment valve

Flow meter

2. Chlorine residual in treated water must be neutralized by the addition of sodium thiosulfate. This is accomplished by using a proportioning injector and a carboy of 2% sodium thiosulfate solution.

Sodium thiosulfate

Proportioning injector adjustment

3. Depending on the circumstances, policies, and regulations for individual utilities, appropriate provisions should be made for directing and disposing of the filtered water. The waste stream should be tested to ensure residual chlorine has been effectively neutralized.

   In a 15 mL tube (VWR #21008-103), add 10 mL water and the contents of a DPD Total Chlorine Reagent pillow pack (Hach, Permachem Reagents Cat. #21056-69). Mix to dissolve. If chlorine is present in the water (content of the tube turns pink), adjust the proportioning injector to increase the sodium thiosulfate until the waste stream tests negative for chlorine (no color change in the contents of the tube).

Waste stream

4. A temperature sample (200 mL bottle of sample water) will be collected at the same time and stored and shipped with the filters as a travel blank.

5. Be sure to record all relevant physical and chemical data (pH, turbidity, temperature, volume filtered, etc) on the sample collection sheet.

6. After samples are collected, store filter capsules at or below 10°C (do not allow capsule to freeze).

7. Disassemble apparatus when filtration is completed. Drain excess water from all tubing and flow meters.

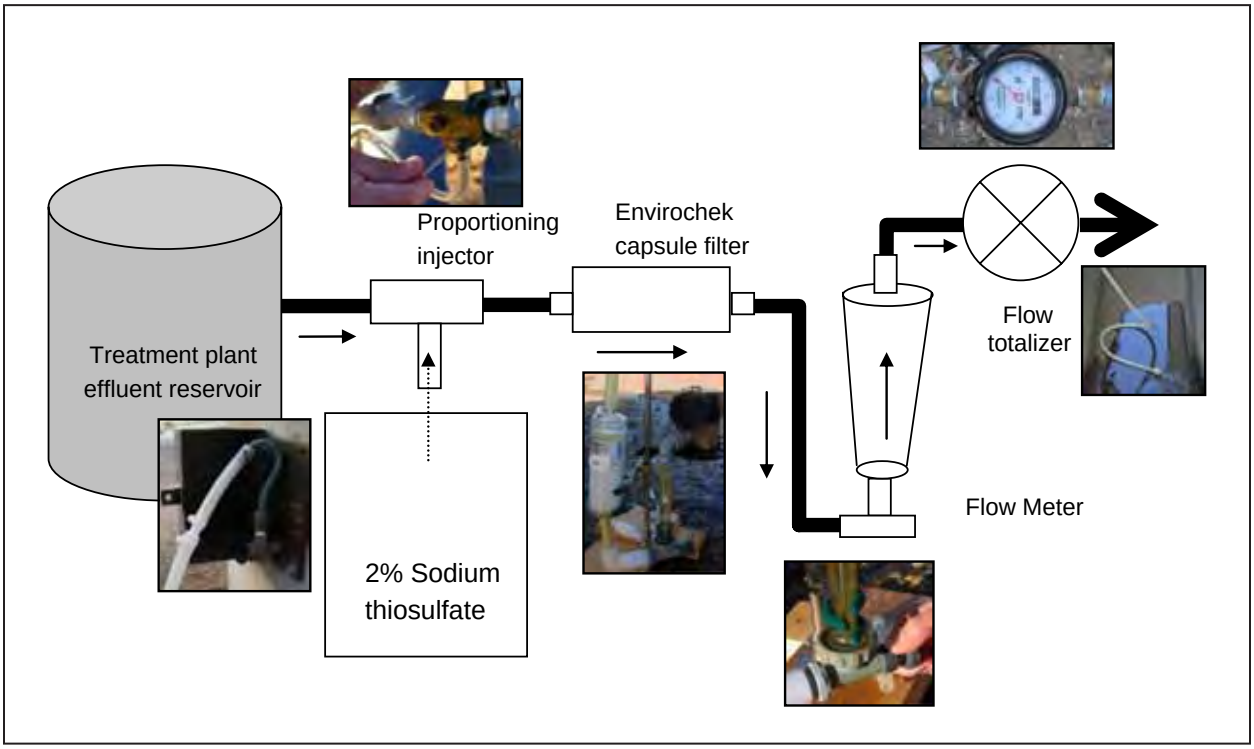
8. Ship filters in coolers provided via overnight courier. Include the following items:

   - Filter
   - Chain of Custody form
   - Sample collection sheet
   - Blue ice packs
   - Temperature monitoring sample

Sample collection sheet

The receiving laboratory will immediately check the temperature sample to verify that it is not over 10°C.

SCHEMATIC OF SAMPLE FILTRATION APPARATUS



QA/QC FOR SAMPLE COLLECTION

A. Method Blanks

a. This will be a 10 L sample of reagent water.
b. A method blank will be run at least once a week or whenever samples are processed, whichever is greater.

B. IPR (Initial Precision and Recovery)

a. At least 4 filters will processed with 990 L of filtered water plus 10 L of spiked water and recovery efficiencies determined.
b. A method blank will also be done.
c. Utilities supplying their own filters will provide recovery information for each lot of filters used.

C. Matrix Spikes

a. For each water source, an initial sample will be filtered (990 L) and then spiked in the laboratory.
b. Matrix spikes will be repeated semi-annually. Alternately, the samples will be analyzed for recovery efficiency and infectivity.

APPENDIX B
DETAILED PROCESSING AND INFECTIVITY PROCEDURE

1. Sample collection and storage:

1.1 Connect the inlet end of the sampling hose to a pressurized tap. Flow rates for filtration can range from 0.7 L/min (total run time approx. 24 hours) to 4 L/min (total run time approx. 4.2 hours) to facilitate the schedules of the utilities and their employees. Do not exceed 30 psi or 4 L/min or the filter integrity may be compromised (use a pressure regulator upstream of the filter if necessary). Adjust the flow rate as necessary by opening or closing the flow meter valve. (1,000 L will be filtered for regular samples and 990 L will be filtered for matrix spike samples.)

1.2 Chlorine residual in treated water must be neutralized by the addition of sodium thiosulfate. This is accomplished by using a proportioning injector and a carboy of 2% sodium thiosulfate solution.

1.3 Depending on the circumstances, policies, and regulations for individual utilities, appropriate provisions should be made for directing and disposing of the filtered water. The waste stream should be tested to ensure residual chlorine has been effectively neutralized.

1.4 After samples are collected, store filter capsules at or below 10°C (do not allow capsule to freeze).

1.5 Disassemble apparatus when filtration is completed. Drain excess water from all tubing and flow meters.

2. Pretreatment of filters with 5% sodium hexametaphosphate (HMP):

2.1 Drain remaining water out of filter from the **outlet** port. You may need to use a pump for this.

2.2 Fill the filter capsule with enough HMP to cover the filter media.

2.3 Shake on wrist-arm shaker for 5 min.

2.4 Immediately drain HMP out of filter from the **outlet** port. You may need to use a pump for this. NOTE: Remove HMP from filter as soon as possible!

2.5 Fill the filter capsule with enough deionized water to cover the filter media.

2.6 Shake by hand.

2.7 Immediately drain the water out of the filter from the **outlet** port. You may need to use a pump.

2.8 Start the Modified Method 1623 elution.

3. Elution of sample: Modified Method 1623

3.1 Elution

3.1.1 Load filter capsule with 125 mL* Method 1623 elution buffer. Agitate on a wrist-arm shaker for 5 min at 900 rpm with the vent cap at the 12 o'clock position (vertical, straight up).

3.1.2 Decant elution buffer into 225 mL centrifuge bottle. Refill filter capsule with 100 mL* Method 1623 elution buffer. Align vent cap at the 4 o'clock position. Shake for 5 min at 900 rpm.

3.1.3 Place vent cap at the 8 o'clock position and agitate on a wrist-arm shaker for 5 min at 900 rpm.

3.1.4 Decant buffer into the centrifuge bottle. Remove top and bottom caps and allow to stand inverted over the centrifuge bottle for at least 15 min.

3.2 Sample concentration

3.2.1 Centrifuge the 250 mL centrifuge tube containing the capsule filter eluate at 2000 × g, for 15 min, at 20°C, no brake. Allow centrifuge to coast to a stop.

3.2.2 Aspirate using a vacuum set at no more than 5 in. Hg. Vacuum down the center of the centrifuge bottle, keeping the pipette tip as far as possible from the sides and bottom of the bottle. Aspirate down to 30 mL mark with vacuum. Use a Pasteur pipette with very light suction to aspirate off the supernatant to the 5 mL mark.

3.2.3 Using a 10 mL pipette pre-rinsed in elution buffer, completely aspirate the resuspended pellet from the centrifuge bottle and measure the volume.

3.2.4 Transfer to a flat-sided Leighton tube containing 1 mL of SL-buffer-A and 1 tmL of SL-buffer-B (see preparation below, 3.3.1.1 and 3.3.1.2).

3.2.5 Subtract the measured volume from ten and divide by two. Use this resultant volume to rinse the centrifuge bottle twice with reagent water. Empty rinse into Leighton tube. Do not allow the total volume of the Leighton tube to exceed 10 mL.

3.3 Sample separation (Purification): Dynal IMS procedure

3.3.1 Add 1 mL of the 10× SL-buffer-A (as supplied, not diluted) to flat sided Leighton tube.

3.3.2 Add 1 mL of SL-buffer-B (as supplied, magenta solution) to the sample tube containing the 10× SL-buffer-A.

3.3.3 Prepare a 1× dilution of SL-buffer-A from the 10× SL-buffer-A (clear, colorless solution) supplied. Use reagent water as the diluent. A volume of 1.5 mL of 1× SL-buffer-A will be required per sample.

3.4 Oocyst capture

3.4.1 Quantitatively transfer the water sample concentrate from section 2 to the flat sided Leighton tube containing the SL-buffer. Label the tube with the sample number.

3.4.2 Vortex the Dynabeads® *Cryptosporidium* vial from the IMS kit for about 10 s to resuspend the beads. Ensure that the beads are fully resuspended by inverting the tube and seeing that there is no pellet at the bottom.

*These volumes are for Falcon 225 mL centrifuge bottles only. A larger volume may be sued for larger capacity bottles.

3.4.3 Add 100 µL of the resuspended Dynabeads® *Cryptosporidium* beads to the flat-sided Leighton tube containing the water sample concentrate and SL-buffer.

3.4.4 Affix the sample tube to a rotating mixer and rotate at approximately 18 rpm for 1 hour at room temp.

3.4.5 Remove the sample tube from the mixer and place the tube in the magnetic particle concentrator (MPC-6) with the flat side of the tube towards the magnet.

3.4.6 Without removing the sample tube from the MPC-6, place the magnet side of the MPC-6 downwards, so the tube is horizontal and the flat side of the tube is facing down.

3.4.7 Gently rock the sample tube by hand end-to-end through approximately 90°, tilting the cap-end and base-end of the tube up and down in turn. Continue the tilting action for two min with approximately on tilt per second.

3.4.8 Ensure that the tilting action is continued throughout this period to prevent binding of low mass, magnetic, or magnetizable material. If the sample in the MPC-6 is allowed to stand motionless for more than 10 sec, repeat step above before continuing to next step.

3.4.9 Return the MPC-6 to the upright position, the sample tube vertical, with the cap at the top. Immediately remove cap and pour off all supernatant from the tube (held in the PMC-6) into a suitable container. Do not shake the tube and do no remove the tube from the MPC-6 during this step.

3.4.10 Remove the sample tube from the MPC-6 and quantitatively transfer the sample to a 1.7 mL microfuge tube with three rinses, using 0.5 mL of 1× SL-buffer-A for the rinses. Liberally rinse down the sides of the flat-sided Leighton tube with the rinses.

3.4.11 Allow the tube to sit for 1-3 min to allow any additional liquid to run down the sides of the tube and transfer it to the microcentrifuge tube.

3.4.12 Place the microcentrifuge tube into the MPC-M magnetic particle concentrator with the magnetic strip in place.

3.4.13 Without removing the microcentrifuge tube from the MPC-M, gently rock/roll the tube through 180° by hand. At the end of this step, the beads should produce a distinct brown dot at the end of the tube.

3.4.14 Immediately aspirate the supernatant from the tube and cap held in the MPC-M. If more than one sample is being processed, conduct three 90° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. (*Note: Do not shake the tube. Do not remove the tube from the MPC-M during these steps.)*

3.4.15 Rinse the IMS sample pellets using 1.0 mL of 1× PBS to minimize debris carryover. In addition to removing debris from samples, this wash step helps remove traces of IMS buffer which can interfere with the pretreatment of oocysts for cell culture.

3.5 Dissociation of magnetic oocyst:bead complex with AHBSS/trypsin

3.5.1 Resuspend the oocyst:bead complex in 200 µL of freshly prepared acidified Hanks balanced salt solution pH 2.0 (AHBSS/trypsin, Sigma Cat. #H9269, with 1.17 mL of 1 M HCl added) containing 1% w/v porcine pancreas type II-S trypsin (Sigma T7409).

3.5.2 Vortex IMS samples and positive control for 10 sec, then incubate for 1 hour at 37°C, vortexing every 15 min.

3.5.3 Immediately after the last vortexing place tubes into the MPC-M with magnetic strip. Transfer supernatants containing dissociated oocysts to labeled microfuge tubes using a P200 micropipettor. If the beads are trailing slightly down the tube wall, tilt the MPC-M slightly away from you while transferring supernatants.

3.5.4 Perform a second wash to remove remaining oocysts from the beads by resuspending the beads in 100 µL of AHBSS/1% trypsin, vortex on high speed for 10 sec, separate with the MPC-M as above and pool samples supernatants.

3.5.5 Add 300 µL of prewarmed (37°C) IFA growth medium without trypsin to samples and positive control. Centrifuge all samples at 15,000 rpm, 2 min, room temperature, no brake, and immediately and carefully aspirate down to 50 µL.

3.5.6 Perform a second wash of all samples by adding 500 µL of prewarmed (37°C) IFA growth medium without trypsin. Centrifuge as above and aspirate down to 20 µL. Resuspend samples in 380 µL (total volume 400 µL) prewarmed IFA growth medium by gentle up and down pipetting and scraping the tube wall using a P200 tip. There should be no clumps. Avoid making bubbles and over-pipetting.

4. Infection of monolayers:

4.1 Obtain 8-well chamber slides with HCT-8 cells that are at least 80% confluent (see section 7).

4.2 Remove the maintenance medium from each cell culture chamber without disturbing the monolayer. Immediately add 100 µL prewarmed growth medium.

4.3 Inoculate each well with the entire sample. The final volume in each well should be 500 µL.

4.4 Incubate at 37°C for 64–72 hours in a 5% CO_2 humidified incubator.

5. Staining of monolayers: After 64–72 hours, the monolayers are stained to detect infectious foci.

5.1 Remove the medium from the wells.

5.1.1 Mock control well: Add the mock control oocysts directly to the monolayer after the removal of the medium.

5.1.2 Immediately add the methanol (Step 5.2) to the monolayer.

5.2 Add 0.8 mL of methanol to each well and incubate 10 min.

5.3 Remove the methanol from the wells.

5.4 Then remove the chambers from the slides using the manufacturer's instructions and tool provided. (Go slowly or the slide will break).

5.5 Place slide in a small tray and pour in the blocking buffer (PBS, 2% goat serum, 0.002% Tween-20), incubate for 30 min. at room temperature.
5.6 Remove the blocking buffer by pouring off into waste beaker.
5.7 Place slides into a humidified chamber.
5.8 Add the rat anti-sporozoite antibody (Waterborne Cat #A600, unlabeled) diluted in 1× PBS. The appropriate antibody dilution was determined for each lot (1:500 dilution was used in this study).
5.9 Incubate for 45 min. at room temperature.
5.10 Was 4 times in 1× PBS. Disperse the 1× PBS over the slide by gently rocking the slide 10 times.
5.11 Place slides back into the humidified chamber.
5.12 Place the secondary antibody, goat anti-rat IgG FITC labeled antibody (Sigma F6258, diluted 1:150 in 1× PBS), onto the slides.
5.13 Incubate for an additional 45 min.
5.14 Remove the antibody and wash 4 times with 1× PBS. Gently rock the 1× PBS over the slide 10 times.
5.15 Put slides on a paper towel and allow to dry.
5.16 Coverslip slides using Waterborne mounting medium (Cat. No. M101).

6. Counting infectious foci:

6.1 Observe the IFA stained monolayers under an epifluorescence microscope equipped with an excitation and emission wavelength filters of 485/520 nm and 515–565 nm, respectively.
6.2 Infectious foci will fluoresce bright green.
6.3 A positive infection for this project was defined as a monolayer with at least 1 infectious focus (3 or more life stages within an approximately circular area ≤175 µm in diameter).

7. HCT-8 cell culture: Cell culture infectivity with HCT-8 cells can be accomplished by several different means. HCT-8 cells can be maintained in-house, prepared slides of confluent HCT-8 cells ready for infection can be purchased, or filtration samples can be sent out to a contract laboratory for processing.

7.1 In-house cell culture method: Monolayers of the human ileocecal adenocarcinoma cell line HCT-8 cells (ATCC CCL-244; American Type Culture Collection, Rockville, MD). Stock cells were maintained in 150 cm^2 flasks and passed twice a week in cell culture maintenance medium. Cells were not used beyond passage 30. Separate biological safety cabinets and incubators were used for uninfected stock cells and the infected monolayers.
7.2 Media Formulations:
7.2.1 *Maintenance Medium*

RPMI-1640 plus GlutaMax (Invitrogen)
5% heat inactivated FBS (Hyclone)
20 mM HEPES
100 U/mL penicillin
100 µg/mL streptomycin
0.25 µg/mL amphotericin B

7.2.2 *IFA Growth Medium*

RPMI-1640 plus GlutaMax (Invitrogen)
10% heat inactivated FBS (Hyclone)
20 mM HEPES
100 U/mL penicillin
100 µg/mL streptomycin
0.625 µg/mL amphotericin B
100 µg/mL kanamycin

7.3 Preparation:

7.3.1 Warm tissue culture medium, PBS, and trypsin to 37°C in a water bath.

7.3.2 Place racks, tubes, flasks, and waste media beaker in a biosafety cabinet and expose to germicidal UV irradiation for at least 30 min.

7.4 Starting new cell passage:

7.4.1 Defrost cell vial from liquid nitrogen.

7.4.2 Add cells to a 75 cm^2 flask and bring volume up to 25 mL with maintenance medium.

7.4.3 When cells become confluent, begin processing as stock cells. New cells must go through 2 passages and mycoplasma testing before using for infection.

7.5 Processing cells for stock cultures:

7.5.1 Remove flasks to be processed from the incubator and place in a biosafety cabinet.

7.5.2 Remove medium, PBS, and trypsin from the water bath, wipe bottles down with 70% ethanol, and transfer to a biosafety cabinet.

7.5.3 Remove the medium from the cell culture flasks and transfer it to the waste beaker.

7.5.4 Add 10 mL of PBS to the flask and gently rock the flask back and forth to rinse the old medium off the cell monolayer.

7.5.5 Remove the PBS from the flask and discard in the waste media beaker.

7.5.6 Add 10 mL of trypsin to the flask. Cap the flask tightly, place the flask into the plastic bag and return it to the incubator for 5 min.

7.5.7 To release the cells from the flask surface, firmly slap the side of the flask.

7.5.8 Transfer the detached cells to a sterile centrifuge tube containing an equal volume of cell culture medium.

7.5.9 Centrifuge the sample for 5 min at 1000 rpm.

7.5.10 In the biosafety cabinet, carefully pour off supernatant while not disturbing the pellet.

7.5.11 Resuspend the pellet in fresh cell culture medium.

7.5.12 Perform a cell count on the cell suspension using a hemocytometer.

7.5.13 Transfer the appropriate amount of cells to a new flask with fresh medium.

7.5.13.1 For a 150 cm^2 flask: 50 mL with 4×10^6 cells.

7.5.13.2 For a 75 cm^2 flask: 25 mL with 2×10^6 cells.

7.5.14 Tighten the cap on the flask, place the flask in a plastic bag, and place the flask in the incubator.

7.6 Processing cells for infectivity:

7.6.1 To set up cells for the infectivity assay, the stock flask was split into two 150 cm^2 flasks in the maintenance medium, the assay flask to be used to set up 8-well chamber slides (IFA assay) and the stock flask to be kept for continued passaging of the stock cells. The assay flask was seeded with 5×10^6 cells per flask so that it would reach 80–100% confluence after 2 days.

7.6.2 Do steps in 7.5 above.

7.6.3 Inoculate HCT-8 cells into 8-well chamber slides (Lab-Tek II, Cat. No. 154534) at a concentration that would allow them to be 80–100% confluent after two days (5.0×10^4 to 4.0×10^5 cells per well).

7.7 QA/QC of media and stock cells: A strict QA/QC procedure should be followed to ensure the health and integrity of the cells used for infectivity throughout the project. The complete cell culture medium was tested for sterility before use.

7.7.1 Media QC:

7.7.1.1 For each bottle of cell culture medium, inoculate 1 mL into each QC medium.

7.7.1.2 When inoculating brain heart infusion broth (BHI) and thioglycollate broth, transfer inoculums, tighten the caps, and invert 2–3 times to mix.

7.7.1.3 After inoculating Sabaroud-Dextrose agar (SDA) and blood agar (BAP), swirl the plates so that the medium is evenly spread throughout the plate.

7.7.1.4 Incubate the QC media for 5 days, while checking daily for growth.

7.7.1.5 If any batch of prepared medium tests positive for bacterial or fungal growth, discard the medium along with any cells that were grown in it.

7.7.2 Mycoplasma testing procedure: Cells are tested for the presence of mycoplasma before use whenever a new lot of cells is thawed.

7.7.2.1 When the cells are passaged for the first time, an aliquot of cells is set up in the medium without antibiotics and passaged twice to allow for the maximum growth of mycoplasma, if present.

7.7.2.2 The cell monolayer is tested for the presence of mycoplasma. Mycoplasma testing can be done in the laboratory with antibodies or DNA analysis with commercially available kits. Cells can also be sent out to a contract laboratory for testing.

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ABBREVIATIONS

AHBSS/T	acidified Hanks balanced salt solution containing 1% trypsin
AIDS	acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BAP	blood agar plate
BHI	brain heart infusion broth
BSA	bovine serum albumin
°C	degrees Celsius
$CaCl_2$	calcium chloride
CC-IFA	cell culture-immunofluorescence assay
CC-qPCR	cell culture-quantitative polymerase chain reaction
CC-RT-PCR	cell culture-reverse transcriptase-polymerase chain reaction
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CI	confidence interval
CISH	colorimetric in-situ hybridization
cm^2	square centimeters
CO_2	carbon dioxide
CV	coefficient of variation
DAPI	4', 6'-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	AgriLife El Paso
F	forward (when used in conjunction with a primer name)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gravitational force
GAC	granulated activated carbon
gpm	gallons per minute
GP60	glycoprotein gene (60 kDa)

h	hour
HBSS	Hanks balanced salt solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
HMP	sodium hexametaphosphate
hsp 70	heat shock protein gene (70 kDa)
ICR	Information Collection Rule
ID_{50}	50% infectious dose
IFA	immunofluorescence assay
IFA-MPN	immunofluorescence assay-most probable number
IgG	immunoglobulin G
IMS	immunomagnetic separation
kDa	kilodalton
kGy	kilogray
$KMnO_4$	potassium permanganate
KU/mL	Kunitz units per milliliter
L	liter
Log_{10}	base 10 logarithm
LT2ESWTR	Long Term 2 Enhanced Surface Water Treatment Rule
M	molar
$MgCl_2$	magnesium chloride
MGD	million gallons per day
mg/mL	milligrams per milliliter
min	minute
mJ/cm^2	millijoules per square centimeter
mL	milliliter
mM	millimolar
MPC	magnetic particle concentrator
mRNA	messenger ribonucleic acid
MuLV	Murine leukemia virus
MWDSC	Metropolitan Water District of Southern California
µg/L	micrograms per liter
µg/mL	micrograms per milliliter
µm	micrometer
µM	micromolar
N	number
na	not available
NA	not applicable
NaOCl/PAC	sodium hypochlorite / poly aluminum chloride

NaOH	sodium hydroxide
nm	nanometers
NTU	nephelometric turbidity units
OCU	Orange County Utilities, Florida Water Division
OPR	ongoing precision and recovery
P	probability
PAC	Project Advisory Committee
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
psi	pounds per square inch
QA	quality assurance
QC	quality control
r	correlation coefficient
R	reverse (when used in conjunction with a primer name)
R^2	square of the correlation coefficient
RCF	relative centrifugal force
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviation
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDA	Sabaroud-dextrose agar
spp.	species
SSU rRNA	small subunit ribosomal ribonucleic acid
SWTR	Surface Water Treatment Rule
T	temperature
TBE	Tris-borate-EDTA buffer
TDS	total dissolved solids
TE	Tris-EDTA
UDG	Uracil DNA glycosylase
U.K.	United Kingdom
U/mL	units per milliliter
U.S.	United States
USEPA	United States Environmental Protection Agency
UV	ultraviolet

V	volts
W	watts
WSLH	Wisconsin State Laboratory of Hygiene
w/v	weight per volume

Detection of Infectious *Cryptosporidium* in Conventionally Treated Drinking Water

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