Potential molecular tools for assessing the public health risk associated with waterborne Cryptosporidium oocysts

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The use of multiple barrier stages at water and wastewater treatment facilities allows for the effective removal of the vast majority of coliforms and other enteric and non-enteric microbes. Subsequent disinfection steps (chlorine, ozone and UV irradiation) are utilized to inactivate microbes that escape the preceding treatment stages. Most viruses, bacteria and protozoa, such as Giardia, are effectively inactivated by chlorination; however, Cryptosporidium is relatively more resistant to environmental conditions and to chlorination. Therefore, UV disinfection has been introduced at many water and wastewater treatment plants to increase log inactivation. Any accidental treatment failure may pose a significant risk to public health. Waterborne transmission of coccidian parasites such as Cryptosporidium and Giardia continues to be a major public health concern. No effective therapies currently exist to treat cryptosporidiosis and the global increase in immunocompromised populations has emphasized the need for water utilities and public health laboratories to have immediate and reliable access to highly sensitive test methods that can determine the host specificity, viability and infectivity of protozoa in the water supply. The most common method used for monitoring Cryptosporidium oocysts and Giardia cysts at intermediate treatment stages and in finished drinking water is the US EPA Method 1623. Although Cryptosporidium species are morphologically indistinguishable, they differ greatly in their host specificity and infectivity. Method 1623 provides quantitative information about Cryptosporidium and Giardia contamination but cannot distinguish between species for intervention purposes in outbreak situations, nor is this method reliable for determining whether the oocyst on the slide is infective for humans. Molecular methods have proven valuable in diagnosing infectious diseases, especially those for which the causative agent is difficult to grow in culture, and similar tools would aid public health agencies to determine risk associated with Cryptosporidium. This review focuses on current methods for determining the host specificity (genotyping), viability and infectivity of Cryptosporidium oocysts.

Introduction

Microbial load is one of the major concerns regarding contaminants in drinking water sources and domestic wastewater. Source water protection and water and wastewater treatment activities are regulated by provincial and federal regulations to ensure that microbial contaminants are effectively removed and/or inactivated to levels that are protective of public health and the environment. In Canada, provincial environmental protection agencies have established stringent limits that are clearly specified in the operating policies and procedures of water and wastewater treatment facilities. These regulatory limits are determined on the basis of the quality of raw intake (raw water supply), considering factors such as microbial loading, population size of town/city and type of treatment facility. Drinking water quality is also regulated by federal guidelines to ensure that it is safe for potable use. Among the regulated bacterial parameters, the most prominent threat for drinking water consumers is posed by E. coli, as opposed to other coliform bacterial groups, such as total coliforms and faecal coliforms (Clark et al., 2010). In the case of wastewater facilities, treatment and disinfection processes (which may include UV irradiation) are regulated to minimize the risk to the environment, aquatic life and downstream users (McCuin & Clancy, 2006; Leone et al., 2009; CCME, 2002).

Abbreviations: CC, cell culture; CC-QSD, cell culture quantitative sequence detection; DAPI, 4',6-diamidino-2-phenylindole; IFA, immuno-fluorescence assay; IMS, immunomagnetic separation; LAMP, loop-mediated isothermal amplification; LCM, laser capture microscopy; NASBA, nucleic acid sequence-based amplification; PI, propidium iodide; SSCP, single-strand conformation polymorphism; qPCR, quantitative real-time PCR; RLH, reverse line hybridization; RT-PCR, reverse transcriptase PCR.
Among the waterborne coccidians, *Giardia* and *Cryptosporidium* are the most common causes of major outbreaks in developed (Steiner et al., 1997) and developing countries (Mak, 2004; Anceno et al., 2007). In the event of treatment failure, *Cryptosporidium* is the primary threat to public health (Harhay et al., 2011; Schuster et al., 2005). Relative to *Giardia* cysts, zoonotic and anthropoontic *Cryptosporidium* oocysts are more resistant to environmental conditions and disinfectants, making *Cryptosporidium* a major risk to public health (Pierce & Kirkpatrick, 2009). The die-off rate of *Cryptosporidium* oocysts in water is $0.005-0.037$ $10^{10}$ log-units day$^{-1}$ under natural environmental conditions (Medema et al., 2006). The potential risk of infection is high for both humans (especially immunocompromised individuals, malnourished children and pregnant women) and animals (particularly pets and livestock in feedlots) (Laupland & Church, 2005; Laupland, 2004; Budu-Amoako et al., 2011). Due to the lack of prophylactic and therapeutic measures against cryptosporidiosis, the mortality rate in humans is an emerging public health issue worldwide (Kothavade, 2011).

*Cryptosporidium* oocysts are ubiquitously present in the environment; thus, humans and other animals can acquire infections through multiple transmission routes (Clark, 1999; Griffiths, 1998). However, there are certain major and minor *Cryptosporidium* species that are directly or indirectly associated with infection in humans. Since *Cryptosporidium* species vary in their host specificity, infectivity and susceptibility to inactivation, determining the risk posed by different *Cryptosporidium* genotypes remains a major challenge to public health agencies. Hence, this review focuses on US EPA method 1623 and the application of current molecular assays for *Cryptosporidium* oocyst genotyping and viability as a measure of host specificity and infectivity. The most recently established molecular techniques are also highlighted for their potential application in detecting a single oocyst from water matrices and rapidly assessing genotype and viability.

**Coccidian protozoans and water treatment**

In areas that utilize source water with low concentrations of *Giardia* and *Cryptosporidium*, such as the Elbow River and Bow River areas in Alberta, Canada, adequate treatment is achieved by filtration and chlorine disinfection. In addition to chlorine, UV disinfection is used in some water treatment facilities that use source water carrying consistently high numbers of *Giardia* cysts and occasionally *Cryptosporidium* oocysts, such as the North Saskatchewan River in Alberta, Canada (Heitman et al., 2002). Although water and wastewater treatment plants are meeting or exceeding regulatory requirements in terms of demonstrating the removal and inactivation of these protozoa, it has been shown that chlorine and UV disinfection regimes may not completely inactivate *Giardia* cysts and *Cryptosporidium* oocysts. The effectiveness of UV disinfection has been demonstrated to be dependent on the protozoan load in the source water supply (USEPA, 2006). Experimental viability studies of *Giardia* cysts (Li et al., 2008) and *Cryptosporidium* oocysts (Belosevic et al., 2001) in water have found that these organisms are still able to transform into their respective trophozoite forms following UV disinfection treatment.

*Cryptosporidium* oocysts have been shown to be resistant to chlorine ($1.05-3.00\%$ chlorine for up to $18$ h) at concentrations $240,000$ times higher than those required to inactivate *Giardia* (Jakubowski, 1995). Therefore, the multiple barrier principle calls for monitoring that may include the watershed, raw intake stage and other serial stages at the water treatment facility, as well as at the post-UV stage to ensure that the treated supply is safe for drinking (USEPA, 2005).

In the case of wastewater treatment facilities, coccidian parasites are released by citizens and pets that have become infected via various modes of transmission from multiple sources (McCuin & Clancy, 2006). The resultant diarrhoeal or defecated material eventually ends up in the wastewater facility for treatment and then is released into the receiving waters (CCME, 2002; USEPA, 2006; USEPA, 1999). Treatment and disinfection processes, which may include UV, are regulated to minimize the risk to the environment, aquatic life and downstream users (McCuin & Clancy, 2006; Leone et al., 2009; CCME, 2002).

**Cryptosporidium species infectious to humans**

The World Health Organization categorizes the coccidian parasite *Cryptosporidium* as a reference pathogen for the assessment of drinking water quality (Medema et al., 2006). Molecular techniques and epidemiological investigations of cryptosporidiosis indicate that, out of the 16 species of *Cryptosporidium*, the vast majority of human cases are caused by *Cryptosporidium hominis* and *Cryptosporidium parvum*, the latter being infectious to both humans and animals (Griffiths, 1998; Sunnotel et al., 2006a; Morgan-Ryan et al., 2002; Paziewska et al., 2007). Such analytical and diagnostic algorithms have been used to identify the infective species in patients with cryptosporidiosis (Cacciò, 2005). Other *Cryptosporidium* species of animal origin, including *Cryptosporidium meleagridis*, *Cryptosporidium suis*, *Cryptosporidium felis* and *Cryptosporidium canis*, have been detected in humans, suggesting a risk of zoonotic transmission (Cama et al., 2007; Llorente et al., 2007; Xiao et al., 2001, 2004) (Fig. 1). One recent *Cryptosporidium* oocyst infectivity study suggests that *C. meleagridis* is infectious to healthy human volunteers (Chappell et al., 2011). *C. meleagridis* is a distinct species that occurs worldwide and, like the *C. parvum* strain, known as the calf or bovine strain, has a wide host range, including cattle. As such, grazing animals may pose risk to surface waters, and because birds are also susceptible to *C. meleagridis*, as well as to some zoonotic strains of *C. parvum*, *C. baileyi* and other *C. parvum*-like parasites (Sréter et al., 2000; Fayer, 2004; Chappell et al., 2011), these animals may also play a role in contamination of surface waters. The most recent
data on major and minor zoonotic and anthroponotic Cryptosporidium species and related clinical case reports are summarized in Table 1.

Infections vary depending on the species involved, but on average, 9–2066 oocysts is the dose required to cause an infection in 50% of subjects (ID50). Of the species that commonly infect humans, this dose is estimated to be 10–83 for C. hominis and 132 for C. parvum (Chappell et al., 2006; DuPont et al., 1995; Okhuysen et al., 1998). Although the lowest infectious dose has been calculated to be 10 oocysts, in reality, one oocyst could be sufficient to cause infection in humans through direct or indirect routes of transmission (Chappell et al., 1996; FDA, 2009). Most importantly, the microscopic features of recovered Cryptosporidium oocysts are uninformative for species identification. Hence, it is essential to have a sensitive and specific genotyping method for determining the host specificity, viability and quantitave risk for cryptosporidiosis.

Isolation, identification and enumeration methods

Application of US EPA method 1623 and associated challenges

High turbidity due to soil or algae in the water matrix is the major contributing factor to the poor recovery of cysts and oocysts from the sample concentrate (pack pellet) after filtration and centrifugation of the water sample (Fig. 2). Larger pack pellets also compromise sensitivity of the method and increase the cost of labour and other expenditures. Despite continual improvements in US EPA methods (ICR A1622A1623) to increase recovery of cysts and oocysts from highly turbid samples, the data generated by this method remain suspect (Krometis et al., 2009). It has, thus, become imperative to improve the most difficult stage of US EPA method 1623, i.e. recovering the entire oocyst population present in the water matrix. Several researchers have dedicated their technical efforts to achieving this goal but have had limited success to date. Contributing to this

Table 1. Clinical cases of cryptosporidiosis from anthroponotic and zoonotic sources

<table>
<thead>
<tr>
<th>Source/reference</th>
<th>Cryptosporidium species</th>
<th>Reservoir</th>
<th>Clinical cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most commonly encountered in humans</td>
<td>C. hominis (anthroponotic)</td>
<td>Humans</td>
<td>Cryptosporidiosis Haiti: contaminated water is a major risk for HIV/AIDS patients, pregnant women and young children</td>
</tr>
<tr>
<td>Raccurt et al. (2006); Llorente et al. (2007); Neira et al. (2010)</td>
<td>C. parvum (zoonotic)</td>
<td>Humans, cattle, sheep and other animals</td>
<td></td>
</tr>
<tr>
<td>Rarely encountered in humans</td>
<td>C. meleagrisid</td>
<td>Turkey</td>
<td>Reported in HIV patients</td>
</tr>
<tr>
<td>Raccurt et al. (2006); Xiao et al. (2002, 2004)</td>
<td>C. felis</td>
<td>Cat, human</td>
<td>Reported in HIV patients</td>
</tr>
<tr>
<td></td>
<td>C. canis</td>
<td>Dog, human</td>
<td>Infection in children and adults from asymptomatic dog</td>
</tr>
<tr>
<td></td>
<td>C. muris</td>
<td>Mouse</td>
<td>In a human in the Western hemisphere, USA, and HIV-positive and healthy persons in Kenya, France, Thailand and Indonesia</td>
</tr>
<tr>
<td></td>
<td>Cryptosporidium spp. cervine genotype</td>
<td>Cervine</td>
<td>Genotyped parasites from the faecal specimens of sporadic cryptosporidiosis cases in British Columbia, Canada</td>
</tr>
<tr>
<td>Pig genotype</td>
<td>Pig/human</td>
<td>Recovered from immunocompetent individual</td>
<td></td>
</tr>
</tbody>
</table>
for the detection of fluorescent circular (oocysts) and egg-shaped (cysts) features and enumeration. 4',6-Diamidino-2-phenylindole (DAPI) staining assists in examining the typical nuclear bodies, and differential interference contrast microscopy is used for the confirmation of internal morphological features. Accurate enumeration is hampered by algae, which mimic the morphology and fluorescence of Cryptosporidium species (Fig. 3; Table 2). The presence of cysts or oocysts on the slide leads to questions about the source, viability and infectivity, i.e. what is the risk and is it infectious to humans? US EPA method 1623 cannot address these questions because this method is not ideal for determining the viability or infectivity of cysts and oocysts. Historically, the presence of internal morphological features within cysts or oocysts has been used as an indication of their viability and hollow cysts or oocysts are still considered to be nonviable but fluorescent staining techniques, such as DAPI inclusion, propidium iodide (PI) exclusion and other nucleic acid stains, are also used for determining the viability and infectivity of cysts and oocysts (Campbell et al., 1992). However, dye permeability and in vitro excystation procedures can overestimate the viability and potential infectivity of treated/disinfected oocysts (Jenkins et al., 1997).

**Significance of genotyping of Cryptosporidium oocysts**

Molecular methods are not routinely applied for species identification in environmental and clinical laboratories; however, these techniques do allow public health services to identify the host-specificity of the recovered oocysts and the associated risk to humans depending on their mode of transmission (zoonotic and anthropotonic species),
determine the source and significance of infections in epidemiological surveillance studies, and distinguish species for intervention purposes, particularly in outbreak situations. Genotyping of Cryptosporidium is useful in determining its public health risk in watersheds. This information may then be used for (i) land-based intervention; (ii) risk-based downstream intervention; (iii) cost-effective approaches in dealing with watershed management, emergency responses and multi-barrier procedures at treatment facilities; and (iv) providing early warning to water and health authorities about Cryptosporidium epidemiology, particularly during extreme weather event scenarios. Accurate genotyping results have demonstrated a distinction between human pathogenic and non-pathogenic genotypes to water authorities, preventing undue alarm when Cryptosporidium oocysts are found in water sampled from protected watersheds. Sending samples to an appropriate molecular biology lab is an ideal approach for genotyping and subtyping; however, such laboratories should be either accredited reference laboratories or laboratories that conduct inter-laboratory comparisons of their methods to maintain accurate performance.

As a result of the significant progress made in developing molecular tools, over 40 genotypes of Cryptosporidium have been identified (Xiao et al., 2004), some of which vary with respect to their development, disease presentation and response to therapeutic measures (Lim et al., 2011). Identification to the species, assemblage and genotype or subgenotype levels has been accomplished by targeting highly variable genes or unique genes. One classical example of this type of molecular analysis found that both human and bovine isolates of C. parvum were linked to human cryptosporidiosis outbreaks, suggesting that there could be two distinct genotypes that infect humans (Peng et al., 1997). The molecular epidemiological significance of all of the genotypes is still unclear.

Assessing the efficacy of current Cryptosporidium genotyping methods for determining the public health significance of oocysts isolated from watersheds is always a challenging issue for management groups and public health practitioners. Thus, several inter-laboratory studies have been conducted to address genotyping methods in epidemiological studies (Ferguson et al., 2006). Some reports suggest a consensus on the application of SSU rDNA genotyping methods to assess the risk of Cryptosporidium in watersheds. The rationale behind selecting this gene is that its single-strand conformational polymorphism is valuable for genotyping to the subtype level, increasing the size of the recently developed database, improving accessibility to compatible primers and the ability to analyse large numbers of samples (Ferguson et al., 2006). In addition, this molecular approach may be able to distinguish between all known genotypes of Cryptosporidium. However, protozoan geneticists are still in the process of standardizing the methods and genetic nomenclature (Xiao et al., 2004).

The most prominent molecular genotyping methods used for determining the public health significance of oocysts are as follows: (i) nested PCR to detect Cryptosporidium oocyst wall protein (COWP) (Pedraza-Diaz et al., 2001); (ii) immunomagnetic separation (IMS)-PCR assay, which permits the highly sensitive detection of C. parvum oocysts in drinking water samples (Hallier-Soulier & Guillot, 2000); (iii) PCR-RFLP assay to amplify Cryptosporidium parvum oocyst wall protein (COWP) for rapid detection (Leone et al., 2009); (iv) FISH technique using fluorescently labelled oligonucleotide probe (18S rRNA Cry1 probe) for detection of C. parvum oocysts in water samples (Alagappan et al., 2009); and (v) hsp70 and 18S rRNA multiplex PCR, which only permits the differentiation of C. parvum and C. hominis (Di Giovanni et al., 2010). These genotyping methods have been applied with promising results to the host-specificity-based source tracking of Cryptosporidium in watersheds (Jenkins et al., 2010).

Because of the availability of ‘ready-to-use sequencers’, sequencing-based methods are the most suitable when discrimination to just the genotype level is required. For larger volumes, the PCR-coupled single-strand conformation polymorphism (SSCP) method, utilizing nuclear DNA regions of the SSU of rRNA, may be preferable because of its lower cost and may become the default genotyping method. The SSCP method has also been used for identification at the subtype level (Xiao et al., 2004). However, more systematic studies are needed to test its ability to detect all genotypes of Cryptosporidium.

### Evolving molecular techniques

**PCR.** US EPA method 1623 and PCR are limited in that they do not provide information about the viability and infectivity of Cryptosporidium oocysts. PCR requires a minimum of 10 oocysts on the slide for effective DNA extraction and subsequent analysis. In addition, slides that are negative for Cryptosporidium by visual examination occasionally test positive by PCR. The viability and infectivity of the oocysts cannot be determined by either method.

For PCR-based analyses, the most commonly used Cryptosporidium-specific biomarker is the gene encoding the 18S rRNA subunit, which has been used for the

### Table 2. Algae species commonly mistaken for Cryptosporidium by microscopy after IFA (Kothavade, 2011, unpublished data)

<table>
<thead>
<tr>
<th>Species</th>
<th>Size (μm)</th>
<th>Shape</th>
<th>Relative fluorescence (Cryptosporidium = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pectodictyon cubicum</em></td>
<td>6</td>
<td>Round</td>
<td>1</td>
</tr>
<tr>
<td><em>Dictyosphaerium pulchellum</em></td>
<td>4</td>
<td>Round</td>
<td>1</td>
</tr>
<tr>
<td><em>Tetraspora lubrica</em></td>
<td>5</td>
<td>Round</td>
<td>2</td>
</tr>
<tr>
<td><em>Cylindropermum</em></td>
<td>2</td>
<td>Round</td>
<td>1</td>
</tr>
</tbody>
</table>
DNA of excysted sporozoites of Cryptosporidium (Hadfield et al., 2011). Other markers, including Cryptosporidium 60 kDa glycoprotein (gp60), microsatellite locus 1 (ML-1) and 2 (ML-2), Cryptosporidium heat-shock protein (HSP70), β-tubulin and Cryptosporidium oocyst wall protein (COWP) have also been used for this purpose (Hadfield et al., 2011; Burton et al., 2010; Ahmed et al., 2010). Although the direct use of PCR does not distinguish between live and dead oocysts, it can be used to detect the DNA of excysted sporozoites of Cryptosporidium oocysts after incubation in excystation medium. The viability of Cryptosporidium is then determined by amplification of an 873 bp region of a 2359 bp DNA fragment encoding a repetitive oocyst protein. This method is adequate to detect low numbers of viable oocysts, such as during routine monitoring of drinking water and environmental samples (Wagner-Wiening & Kimmig, 1995).

DNA hybridization is the most important stage in the PCR; hence, the amount of high-quality DNA is very important. Although PCR is an efficient tool for tracking the source of community-acquired diseases and for epidemiological and transmission studies, its specificity and sensitivity are commonly affected by the sample matrix, which may contain inhibitory factors that particularly affect the DNA extraction stage. PCR amplification of DNA extracted from Cryptosporidium oocysts has also been found to be inhibited by environmental factors such as formaldehyde, potassium dichromate and humic acids, which are generally carried over through the extraction and purification stages (Bakheit et al., 2008; Skotarczak, 2009).

**Real-time PCR.** In this technique, the quantity of DNA product is measured in every cycle of the PCR, i.e. in real-time. Real-time PCR, also called quantitative real-time PCR (qPCR), can be used without an electrophoresis stage. qPCR has demonstrated superiority over conventional PCR because of its ability to detect amplified DNA products within an hour without any environmental contamination risk. Another major advantage of qPCR is that it is compatible with multiplex PCR, which is a cost-effective analytical method. Furthermore, after converting RNA into cDNA with the use of reverse transcriptase, the resulting quantity of RNA can also be estimated by reverse-transcriptase PCR (RT-PCR) (Skotarczak, 2010).

In the fluorescence-based RT-PCR method, the fluorescence emitted from the rapidly increasing number of fluorochrome-labelled DNA molecules is detected in a specialized thermocycler. Alternatively, with the TaqMan method, fluorescent oligonucleotides that are complementary to the amplified DNA sequences are used; such probes emit light under specific conditions. A fluorochrome-carrying probe is used in this method and the fluorescence-suppressing molecule is hydrolysed by Taq polymerase during the elongation stage (Skotarczak, 2010). Varying matrices have been tested with qPCR for the detection and identification of Cryptosporidium oocysts to the species or genotype level. A test of the sensitivity of this analytical method has demonstrated its ability to detect a minimum of three oocysts (Sunnotel et al., 2006b).

**Laser capture microscopy (LCM) in conjunction with qPCR.** The use of LCM in conjunction with qPCR for the detection of Cryptosporidium oocysts provides a rapid, sensitive and highly specific analysis of fixed oocysts on slides (Sunnotel et al., 2006b). However, further studies, particularly on environmental samples, are warranted. Using 18S rRNA as a target, the detection sensitivities of real-time PCR and nested-PCR systems have been evaluated (Sunnotel et al., 2006b; Espy et al., 2006). Some researchers have been able to routinely detect a single oocyst when targeting 18S rRNA (Espy et al., 2006). Furthermore, for distinguishing Cryptosporidium morphotypes on a glass slide, a nested PCR analysis targeting the 18S rRNA locus is a robust and sensitive molecular technique. Although qPCR allows a quicker turnaround time for detection and identification, LCM could be used for genotyping single Cryptosporidium oocysts. This combined molecular approach can also be used for distinguishing between different C. parvum isolates (Espy et al., 2006).

**Reverse line hybridization (RLH).** A traditional RLH assay targeted specifically at Cryptosporidium species is still in the developmental stages. RLH involves an important analytical stage that includes membrane and probe binding. Aminelabelled probes are bound covalently to an activated negatively charged nylon membrane. Biotin-labelled PCR amplicons are then hybridized to a membrane-bound probe. Streptavidin labelled with peroxidase is incubated with the membrane and binds to the biotin-labelled PCR amplicons. The amplicons are then detected by enhanced chemiluminescence. Of the two detection reagents added, one decays to hydrogen peroxide and is reduced by the enzyme peroxidase. The reduction reaction causes the luminol in the second detection reagent to be oxidized (Kong & Gilbert, 2007; Bandyopadhyay et al., 2007). The resulting by-products emit light, which is detected by sensitive photographic film or a compact flow cytometer. The 100% compatibility of the designed probe with microbial amplicons allows the identification of multiple species on the same slide, as well as micro-heterogenetic differences and interspecies variability within the organisms of interest. This method permits accurate species/genotype identification in larger populations (Skotarczak, 2010) and is well suited to the source tracking of Cryptosporidium in watershed protection programs.

**Luminex-based assay as an improved version of RLH.** Continual improvements of RLH have resulted in a new method that eliminates the DNA sequencing step after PCR (Bandyopadhyay et al., 2007). This so-called Luminex-based assay can differentiate between C. hominis and C. parvum in a rapid and cost-effective manner. This assay involves the use of species-specific probes coupled to carboxylated Luminex microspheres that hybridize to a repetitive sequence in the microsatellite-2 region (ML-2) where C.
hominis and C. parvum differ by one nucleotide substitution. The 100% specificity of the assay has been verified by direct fluorescent antibody test (DFA) and DNA sequencing analysis. Moreover, the sensitivity had been found to be superior to that of DFA (Bandyopadhyay et al., 2007).

Loop-mediated isothermal amplification (LAMP) of DNA. Most of the molecular assays described above are not sensitive enough to deliver results with low numbers of Cryptosporidium oocysts. The LAMP assay, however, has been demonstrated to detect organisms at relatively low concentrations in environmental test samples. This extremely specific procedure is able to isolate a DNA template from a sample containing several biological contaminants. Recent studies have clearly shown that the LAMP assay is superior to nested PCR in terms of its accuracy and high sensitivity for Cryptosporidium and Giardia species (Nago et al., 2010; Bakheit et al., 2008).

The LAMP method is based on loop-mediated isothermal amplification. LAMP amplifies DNA rapidly, with high specificity and efficiency. For this assay, DNA polymerase and a set of four specially designed primers (starters) that recognize a total of six distinct sequences on the target DNA are used. The LAMP reaction begins with an inner primer that contains sense and antisense sequences of the DNA template. In the next stage of this reaction, the outer primer initiates the synthesis of separated DNA strands. By using this as a template, further synthesis of DNA is conducted from the second inner and outer primers that hybridize to the other end of the target, which consequently generates a stem–loop DNA structure with a stem twice as long as the template. Because of the continuous cyclic reaction, almost 10^9 DNA copies are accumulated within an hour. The end products are stem–loop DNAs with multiple inverted repeats of the target and cauliflower-like structures with multiple loops that are formed by annealing between alternately inverted repeats of the target in the same strand. These are initially detected during the LAMP reaction by six distinct sequences followed by four distinct sequences (Notomi et al., 2000). The LAMP assay has been used for detecting Cryptosporidium on the basis of amplification of the gp60 and hsp70 genes of C. parvum; however, further fine tuning of this method is required, particularly for species identification and differentiation, which requires an adequate range of species-specific DNA sequences from GenBank. The major advantage of this method is its insensitivity to commonly occurring PCR-inhibiting factors in environmental samples (Notomi et al., 2000; Karanis et al., 2007; Bakheit et al., 2008; Skotarczak, 2009). Evaluation of this method with varying matrices may be the first step towards its application to water quality monitoring programs.

Progress in Cryptosporidium viability analysis

Dedicated researchers have developed methods that permit determination of the viability of environmentally stable cysts or oocysts. TYI-S-33 medium contains acids and enzymes that promote the excystation of Giardia cysts but the assay is not adequate for determining the rate of excystation. C. parvum oocysts can also be excysted as a measure of viability (Diamond et al., 1978; Black et al., 1996). Non-excysted oocysts recovered after commonly used excystation procedures have been found to be infectious to neonatal mice (Neumann et al., 2000). Giardia cysts and Cryptosporidium oocysts can excyst in the gastrointestinal tracts of other animals in addition to humans. Mongolian gerbils have been used as an experimental model for Giardia infection (Belosevic et al., 1983), and neonatal CD-1 mice have been used as a model for Cryptosporidium infection (Finch et al., 1993). However, as well as the problem of needing additional resources, funds and labour, the relevance of the data generated from such studies for risk assessment and for treatment purposes remains dubious. Moreover, caution should be taken in interpreting the results of viability or infectivity assays in mouse or any other animal models, as these results might not necessarily reflect the susceptibility or resistance in humans due to host specificity and immunological differences.

Some simpler and more manageable tissue culture assays have been developed using human ileocaecal adenocarcinoma (HCT-8) cell monolayers. Cysts/oocysts from water samples are concentrated and removed of other contaminants and then inoculated on HCT-8 cells, resulting in successive reproductive stages that can be examined by indirect antigen–antibody testing or RT-PCR (Slikfo et al., 1997; Rochelle et al., 1997). Comparison of this method with others demonstrates a superior over conventional methods. However, a major challenge of in vitro culture methods is the maintenance of a cell line, which is often subject to poor reproducibility among similar samples for quantitative assessments.

RT-PCR is a molecular tool that has also been found to be useful for the direct detection of viable Giardia and C. parvum in water containing concentrated cysts and oocysts. A comparison of RT-PCR and immunofluorescence assay (IFA) and microscopy assay (DAPI/PI) for testing viability suggested that RT-PCR is better for viability detection in Giardia, whereas IFA is superior for detecting viable Cryptosporidium oocysts (Johnson et al., 2012). Other molecular assays, such as FISH and nucleic acid probes, can be used to detect 18S rRNA in Giardia and Cryptosporidium. However, while 18S rRNA can be detected at higher levels in viable cysts/oocysts by FISH and nucleic acid probes, this method is not an ideal representation of their viability (USEPA, 2007). Further studies are needed to fine-tune its detection limit and ability to assess cyst/oocyst infectivity. While several other molecular methods have also been developed, their performance remains unsatisfactory, particularly with respect to detecting potentially infectious waterborne Cryptosporidium oocysts.

Integrated approach: IMS and chemical cycling-PCR

The US EPA Information Collection Rule method (USEPA, 1996) and the proposed method 1622 (USEPA, 1997) for the detection of Cryptosporidium oocysts recovered from
water samples are not suitable for the detection of C. parvum, which is infectious to humans, nor are they suitable for determining viability and infectivity. Multiple PCR-based methods for the detection of C. parvum have been evaluated (Wagner-Wiening & Kimmig, 1995; Stinear et al., 1996; Johnson et al., 1995); these methods include an infectivity assay based on in vitro cell culturing of the parasite with Caco-2 cells and an assay for the detection of C. parvum-infected cells by targeting C. parvum hsp70 mRNA using RT-PCR (Rochelle et al., 1997). Integration of IMS has been used on purified samples of oocysts from water samples. These samples were subsequently added to in vitro monolayers of human ileocaecal adenocarcinoma HCT-8 cells and C. parvum-infected cells were detected by targeting C. parvum hsp70 DNA using standard chemical cycling PCR techniques (Di Giovanni et al., 1999). This standardized method has been used for monitoring Cryptosporidium inactivation by water treatment processes.

Quantitative PCR and in vitro cell culture

The method referred to as cell culture quantitative sequence detection (CC-QSD) uses HCT-8 human ileocaecal adenocarcinoma cell culture in a 96-well plate format and TaqMan PCR targeting the hsp70 gene of C. parvum to determine infectivity or viability (Khramtsov et al., 1995). For the comparison of samples, a CC-QSD standard curve must be generated by inoculating HCT-8 monolayers with different numbers of C. parvum oocysts and performing linear regression of sample mean cycle threshold values against numbers of inoculated oocysts. The CC-QSD method permits determination of the effects of incubation time on the cell culture development of C. parvum, evaluation of the effects of oocyst pretreatment on cell culture infectivity and comparison of different C. parvum isolates. Furthermore, CC-QSD is a less labour-intensive, more flexible alternative for these types of C. parvum cell culture assays compared with previously proposed methods (Di Giovanni & LeChevallier, 2005).

Recent applications of HCT-8 cell monolayers for assessment of Cryptosporidium infectivity

For Cryptosporidium cell culture assays, HCT-8 monolayers are typically allowed to grow for 24 or 48 h and to reach 80–100 % confluence (freshly confluent) just prior to inoculation (Arrowood, 2002; Woods & Upton, 2007). However, this timing can cause difficulties in coordinating water sample collection and processing with readiness of the cell monolayers for inoculation, consequently making it complicated to perform Cryptosporidium cell culture assays on a day-to-day basis. The results of Sifuentes & Di Giovanni (2007) suggest that HCT-8 monolayers as old as 67 days can support an infection. Additionally, repeated experiments with aged monolayers (8–11 days old and 11–22 days old) showed that the aged monolayers developed the same number of C. parvum clusters of infection as baseline 2-day-old freshly confluent monolayers. These results suggest that it may be possible to use cell monolayers that are up to 3 weeks old for infectivity assays without having to schedule sample processing to coincide with the development of freshly confluent monolayers.

Despite dedicated efforts, there has been no success in developing a method to produce mature Cryptosporidium oocysts in cell culture; thus, researchers must still rely on animal propagation methods. The long-term maintenance of Cryptosporidium in cell culture has been reported previously and recent studies have evaluated the effects of oocyst treatment on excystation and host cell growth phase (Kato et al., 2001; Woods & Upton, 2007). Recently, significant progress in the optimization of oocyst pretreatment and cell monolayer growth conditions have been made by using a dual approach of using cell culture in conjunction with IFA to measure total and infectious oocysts (Lalancette et al., 2010). Development and optimization of this method allows for combined detection of total and infectious oocysts, which could be used to improve tools for the assessment of Cryptosporidium-associated risk in the water industry. The dual detection 3D-cell culture-IFA method allows for the calculation of the infectious portion of oocysts by providing a combination of total and infectious counts.

Nucleic acid sequence-based amplification (NASBA)

NASBA is an rRNA-based method that is superior to RT-PCR because it is independent of the presence of DNA in varying water matrices. In NASBA, the isothermal reaction takes place at a temperature of 41 °C and does not require denaturation. Consequently, it avoids amplification of other genomic DNA, which often occurs when testing environmental samples (Skotarczak, 2009; Notomi et al., 2000; Mens et al., 2006). NASBA has prospective applications as a Cryptosporidium detection technique (viable and nonviable oocysts) from environmental samples. Additionally, this technique has been able to differentiate between three species of virulent (pathogenic) and a number of other avirulent Cryptosporidium species (Connelly et al., 2008).

The molecular mechanism of this method involves the initial template RNA along with three enzymes and two primers. Reverse transcriptase amplifies a single cDNA strand starting from one of the primers. Next, RNase H degrades RNA from the resulting RNA–DNA hybrid. The second primer, which is supplemented with a T7 promoter sequence, then hybridizes to the cDNA and transcription begins with the third enzyme and RNA polymerase. Each transcript is a template for creating another cDNA with a T7 promoter, which, in turn, is a template for RNA transcripts (Skotarczak, 2009).

NASBA has also been demonstrated to be an ideal method for detecting the viability of waterborne oocysts. Most interestingly, this method is capable of determining the
viability of immunomagnetically separated oocysts belonging to the species of *Cryptosporidium* that are pathogenic to humans, including *C. parvum*, *C. hominis* and *C. meleagridis* (Connelly *et al.*, 2008). After oocysts are immunomagnetically separated from the water sample (US EPA method 1623), mRNA is extracted with oligo-dT-coupled magnetic beads, amplified via NASBA and then detected in a nucleic acid hybridization lateral flow assay. This method is highly sensitive, with the ability to detect one oocyst in 10 μl using flow cytometer-counted samples. Additionally, it detects only viable oocysts, as demonstrated by DAPI/PI staining. The performance of the NASBA assay has been evaluated for the detection of oocysts in the presence of large numbers of common waterborne micro-organisms and pack-pellet material filtered from environmental water samples. Significantly comparable results were obtained when NASBA was compared with US EPA method 1622 for *C. parvum* detection. Connelly *et al.* (2008) found NASBA to be an ideal method for monitoring the safety of drinking water based on this detection system.

**Cell culture sensitivity and reproducibility**

IFA, PCR and RT-PCR are the most commonly used assays in the water industry that allow the detection of *Cryptosporidium* infection in HCT-8 cells. Recently, Johnson *et al.* (2012) examined these assays for their relative sensitivity, reproducibility and frequency of false positives. Monolayers of HCT-8 cells were exposed to a variety of viable and inactivated oocysts to assess assay performance. All of these assays detected infection with one or three oocysts (enumerated by flow cytometry). Amongst all the assays studied, PCR was found to be the most specific, but with the highest frequency of false positives with mock-infected cells and inactivated oocysts. The most significant performance was demonstrated by IFA due to its efficiency in detecting infection caused by *Cryptosporidium* oocysts without producing any false-positive results, particularly with mock-infected monolayers. IFA has also been advocated for routine and sensitive detection of infectious *C. parvum* and *C. hominis* in drinking water. This suggestion was made on the basis of IFA assay efficiency to detect spiked infectious oocysts recovered from Envirocheck capsules (Pall Corporation) upon filtration of 1000 litres of treated water (Johnson *et al.*, 2012; USEPA, 2005).

**Analysis techniques for the evaluation of viability**

Infectivity or viability of *Cryptosporidium* oocysts has been evaluated by several molecular, culture or infectivity assays such as: (i) evaluation of comparative infectivity in CD1 mice and in cell cultures using RT-PCR detection assay targeting *hsp70* gene mRNA (Rochelle *et al.*, 2002, 2004), (ii) evaluation of infectivity in HC8 cells using qPCR targeting the 18S rRNA gene (Garvey *et al.*, 2010), (iii) cell culture (CC)-PCR assay targeting *hsp70* DNA (Aboytes *et al.*, 2004), and (iv) CC-IFA assay on oocysts recovered by USEPA method 1623 (Quintero-Betancourt *et al.*, 2003; Gennaccaro *et al.*, 2003).

Application of PCR assays, particularly CC-PCR and qPCR, is not encouraged for analysis of infectivity due to their high frequency of false-positive results (Di Giovanni & LeChevallier, 2005). CC-PCR generates higher false positives with inactivated oocysts and mock infections. This assay may underestimate UV inactivation of oocysts due to the amplification of DNA in irradiated oocysts or sporozoites remaining on the cell monolayer (Bukhari & LeChevallier, 2003). In the case of the qPCR assay, background signal due to high inoculum densities is the major concern (Di Giovanni & LeChevallier, 2005). Hence, higher infection rates detected by the PCR assay may be the result of a relatively high frequency of false positives (Johnson *et al.*, 2012). Additionally, growth supplements such as glucose and vitamins used in the inoculum for PCR assays have also been reported as major contributing factors in to increased *C. parvum* cell culture infection. This could be another explanation for higher detection levels for the PCR assays (Upton *et al.*, 1995).

The recent studies suggest that CC-IFA has the most potential as an infectivity assay that can be used on a broad-spectrum pattern of water quality monitoring programs required by regulatory agencies (Johnson *et al.*, 2012). The rationale behind selecting this assay includes: (i) a relatively low number of false positives with inactivated oocysts (one and three oocysts on monolayer); (ii) a relatively better performance than other assays with oocysts recovered from spiked filters using US EPA method 1623; (iii) it being one of the simplest methods with fewest processing steps; and (iv) its sensitivity to predict the number of infectious oocysts (Johnson *et al.*, 2012; Bukhari & LeChevallier, 2003).

Molecular biology expertise, equipment, reagents, proficiency-testing performance and continual improvement become prohibitively expensive for establishing cell culture assays for use in water utility laboratories. Some inherent variability in cell culture-based infectivity assays for *Cryptosporidium* oocysts remains a challenging issue as well.

**Conclusion**

Monitoring contamination levels in the watershed, raw water intake, intermediate treatment stages and finished drinking water is part of the multiple barrier approach to ensure safe drinking water. In the case of wastewater treatment plants, it aids in monitoring treatment efficiencies to minimize the risk to the environment, aquatic life and downstream users. Since the advent of multiple genotyping methods, source tracking of *Giardia Cryptosporidium* in watersheds is becoming easier and aids in the prevention and control of any potential sources of contamination. To a certain degree, the source of protozoan
contamination may suggest whether the species/assemblage identified poses any immediate health risk. However, to be more precise in assessing these risks, it is necessary to develop, in addition to animal infectivity models, molecular tools or in vitro assays to test viability and infectivity.

Although recently studied CC-IFA seems to be a reasonable option for more widespread use of detection assays in the water industry, particularly for assessing the public health risk, the genotyping methods such as NASBA and RLBH are the two potential molecular techniques that need to be standardized in conjunction with RT-PCR. This may be the rational approach for determining accurate identification of the Cryptosporidium species and the source of contamination. Furthermore, these techniques could be aligned with the recently modified assays for viability and infectivity testing. Recently modified IFA and NASBA are potential techniques for use in water laboratories or public health laboratories. However, very meticulous parallel studies are certainly warranted to establish simpler, rapid and cost-effective, yet accurate, analytical algorithms in reference laboratories.

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