Review Paper

A review of the existing methods for detection, enumeration and inactivation of *Cryptosporidium* in surface waters

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ABSTRACT

Assessing the microbiological quality of water for human consumption and/or recreational purposes has been a major concern for water authorities and public health officials for many years. To maintain the health and welfare of water consumers and to ensure public confidence in the water authorities, vigilant monitoring of source and treated water supplies must be undertaken. Part of the requirements for achieving optimal water quality requires the use of highly sensitive, reproducible and reliable microbiological assays to detect the presence of pathogenic microorganisms and their abundances in surface waters. This review paper examined the current detection and enumeration methods for *Cryptosporidium* in water samples. Attempts have also been made to evaluate the current epidemiology of *Cryptosporidium* in relation to water borne outbreaks.

Key words | *Cryptosporidium*, detection methods, enumeration, water borne outbreaks

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BACKGROUND

Cryptosporidium is a protozoan parasite of major public health significance. The main symptom of cryptosporidiosis is watery diarrhoea, which may contain mucus but rarely blood. The severity of the disease can vary greatly, depending on the individual's immune status (Fayer *et al.* 1997; Marshall *et al.* 1997) but is potentially life threatening to immunocompromised individuals (Byleveld *et al.* 1999). The infected human or animal can excrete millions of oocysts in their faeces (Hayes *et al.* 1989; Suwa & Suzuki 2003) which, although they cannot reproduce outside the host, can survive in the environment, especially fresh water, for up to 6 months (Thomas *et al.* 2000). Person-to-person contact and contact with domestic animals are common sources of infection, but exposure to parasites through the use of surface waters for recreational activities or consumption of contaminated drinking water are shown to be important routes of transmission (Fayer *et al.* 1997). There have been many reported outbreaks of cryptosporidiosis due to use of contaminated recreational (Kebabjia 2001; Puech *et al.* 2001) and drinking water supplies (MacKenzie *et al.* 1994; Thwaites 1997). Water treatment plant equipment failures have also been regarded as a potential cause of water supply contamination (Mazounie *et al.* 2000). The most notorious outbreak was in Milwaukee in 1993, where more than 400,000 people became ill with diarrhoea, 4,400 were hospitalized and 69 died (Thwaites 1997). The most recent incident in Australia, known as the 'Sydney Water Crisis', occurred in 1998 when *Cryptosporidium* was detected in the treated water supply (Mazounie *et al.* 2000). Although no incidents of illness were reported, the issuing of boil water alerts was deemed the appropriate response to the incident to protect the health of the population (Stein 2000).

Studies have shown that animal activity in catchment areas poses a major threat to surface water quality (Karanis

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1997; Graczyk *et al.* 2000; Bodley-Tickell *et al.* 2002). Factors such as the direction of run-off, fencing, land use, handling of animal and human waste and hydrology will all affect the transmission of *Cryptosporidium* in the environment (Rose *et al.* 2002). Many water borne outbreaks have occurred during or following heavy rainfall (Rose *et al.* 2002). Parasites may be present in sediment or washed from contaminated land into the waterways. Depending on rainfall events or the activity of animals in the area, sediment could also be disturbed by animal activity on the river bed, or there could be direct depositing of faeces into the water, even when rainfall was low (Atherholt *et al.* 1998; Thurman *et al.* 1998). However, high rainfall does not always coincide with elevated levels of *Cryptosporidium* (Carrington & Miller 1993; Kistemann *et al.* 2002; Wohlsen *et al.* 2006*b*).

The continuous threat of contamination with *Cryptosporidium* in surface waters and treated water supplies has led to the development or improvement of existing detection methods for this parasite in surface waters. Assessments of these methods in practice have shown varying degrees of recovery efficiency (Nieminski *et al.* 1995; Shepherd & Wyn-Jones 1995, 1996; Hsu *et al.* 2001; Simmons *et al.* 2001; McCuin & Clancy 2003; Wohlsen *et al.* 2004). The matrix variation and complexity of the procedures could explain these varying results (DiGiorgio *et al.* 2002).

DETECTION AND ENUMERATION OF *CRYPTOSPORIDIUM* IN WATER

Public health issues

Maintaining the health and welfare of water consumers requires a highly sensitive and accurate microbiological analysis of water samples. To ensure that the quality of water for human consumption and/or recreational purposes is maintained, vigilant monitoring of source and treated water supplies must be undertaken. While detection and enumeration of faecal indicator bacteria have been used by water authorities as a key tool for water quality analysis (NHMRC 2004), detection of specific pathogens may on some occasions be required owing to the lack of a relationship between the presence of pathogens and faecal indicators. It has been shown that the presence of *Cryptosporidium* oocysts in surface waters has been mainly related to farming activities (Atherholt *et al.* 1998; Thurman *et al.* 1998) and the fact that the oocysts can survive for up to 6 months in such an environment has made its detection a major public health issue.

Many methods have been developed to detect *Cryptosporidium* in surface water samples. All methods require the concentration of large sample volumes, typically 10 l or more and are technically demanding. The implications of an incorrect analysis (i.e. false negative or false positive results) of water samples can have public health, political and legal consequences. The common problem with all of the current detection methods is that no method can detect 100% of oocysts or cysts present in a sample. If the concentration method produces a recovery too low, a false negative result may be reported. The fact that national and international accreditation bodies accept percentage recoveries ranging between 10 and 110% (National Association of Testing Authorities, Australia 2002) is an acknowledgement of the difficulty in analysing water samples for the detection of *Cryptosporidium*. False positive results may occur as a result of microscopic misidentification of *Cryptosporidium*- and *Giardia*-'like organisms' (i.e. yeast and some algae). A false positive result may cause unnecessary action by the health authorities (i.e. boil water alerts) and loss of income for businesses relying on the water supply to conduct their duties. However, a false negative result may allow contaminated water to reach the drinking water supply and become a public health risk. This scenario has far greater implications for public health officials and water authorities, both politically and legally. It is for this reason that improvements to the current methods are necessary in order to prevent recovery efficiencies from being so low that false negative results are reported.

Current guidelines and regulations

Worldwide studies have addressed the occurrence of *Cryptosporidium* and *Giardia* in surface waters (Thurman *et al.* 1998; Gasser & O'Donoghue 1999; Wohlsen *et al.* 2006*b*). As a result, national guidelines for monitoring of *Cryptosporidium* have been developed. In the United Kingdom, the Drinking Water Inspectorate (DWI) was the first regulatory body to implement regulations in 1999,

which made it a criminal offence to supply water containing greater than 1 *Cryptosporidium* oocyst in 10 l of treated water (UK, DWI 1999). The Australian National Health and Medical Research Council (NHMRC 2004) has set no guideline value for *Cryptosporidium* and no routine monitoring of distribution systems is recommended. However, the detection of *Cryptosporidium* in the treated water supply in Sydney (Quill 2000) has led to ongoing monitoring surveillance in the state of New South Wales. Both Canada and Australia use a multi-barrier approach to prevent contamination rather than define a guideline value (Health Canada 2004; NHMRC 2004).

Detection methods

The sensitivity of the detection methods for *Cryptosporidium* in water is of great importance. It has been recommended that the method applied for detection of *Cryptosporidium* should ideally be sensitive enough to detect 1 oocyst in 100 l of water, but detecting 1 oocyst in 10 l would be more realistic (Vesey *et al.* 1993). To isolate oocysts from such a large volume of water, different types of membrane filter such as flat-bed cartridge or polycarbonate cartridge are used in most laboratories. Membrane filters are then eluted and further concentrated using either density gradient flotation techniques or immunomagnetic separation of oocysts and cysts. All procedures require immunofluorescent staining using a monoclonal antibody to visualize the oocysts. Some of these methods and supplementary techniques associated with them are described below.

Filtration methods

The most common membrane filters used to concentrate water samples are: pleated membrane capsule filters, flat-bed membrane filters, compressed foam depth filters, ultrafiltration, hollow-fibre filters and yarn-wound cartridge filters (Nieminski *et al.* 1995; Shepherd & Wyn-Jones 1995, 1996; Hsu *et al.* 2001; Simmons *et al.* 2001). These filters have demonstrated varying degrees of recovery efficiency for *Cryptosporidium* oocysts and/or *Giardia* cysts from both surface and treated water samples.

There are currently two types of pleated membrane capsule filter: the Envirochek™ standard and the Envirochek HV™ (high volume); both are made by Pall Corporation (previously Gelman). Using the United States Environmental Protection Agency (USEPA) Method 1623, DiGiorgio *et al.* (2002) demonstrated recovery efficiencies ranging from 37 to 43% for oocysts and from 0.8 to 61% for cysts using the standard filter. With the Envirochek HV™ filter, the recovery for oocysts ranged from 36 to 51% and for cysts from 0.5 to 53%. The lower recoveries for both filters were obtained from water samples with high turbidity readings (i.e. 99 NTU).

Simmons *et al.* (2001) compared ultrafiltration (hollow-fibre) to the Envirochek capsule filter (standard) for the recovery of *Cryptosporidium* in surface waters and found the recovery ranged from 12 to 93% for the ultrafilter and from 4 to 44% for the Envirochek HV™.

Ferguson *et al.* (2004) compared the compressed foam depth filters (FiltaMax) with the Envirochek HV™ and membrane filtration and found that the mean recovery for oocysts was 27% for membrane filtration, 18% for the Envirochek HV™ and 19% for the FiltaMax. For cysts, these values were 42% for the membrane filter, 29% for the Envirochek HV™ and 29% for the FiltaMax. Higher recovery was achieved by McCuin & Clancy (2003) for the FiltaMax, where the mean recovery ranged from 39 to 51% for oocysts and from 25 to 47% for cysts.

Recovery efficiencies for yarn wound cartridge filters (25.4 cm, 1.0 μm pore size) have been demonstrated to range from 9.3 to 9.8% for oocysts and from 28 to 30% for cysts in raw and treated waters, respectively. For membrane filtration (142 mm, 3.0 μm pore size), these values ranged from 16 to 17% for oocysts and from 38 to 40% for cysts (Hsu *et al.* 2001). The matrix variation, turbidity of the samples and complexity of the procedures could explain these varying results between researchers (DiGiorgio *et al.* 2002). Most of these studies demonstrated higher recoveries for *Giardia* cysts than for *Cryptosporidium* oocysts. This could be due to the larger size of the *Giardia* cysts, making it easier for them to be captured by the filter (Falk *et al.* 1998). Furthermore, most seeding solutions are enumerated manually by performing dilutions and microscopically counting the oocysts/cysts to achieve the desired count for the seeding stock solutions. This may also contribute to the existing variation in the number of captured oocysts and/or cysts indicating the need for a uniform approach of

seeding suspensions to ensure fair comparisons between different filtration methods.

Using precise inocula, Wohlsen *et al.* (2006*a*) evaluated the recovery efficiency of five commonly available membrane filtration techniques with and without additional modifications. These were the Envirochek™ and Envirochek HV™ filters, Millipore and Sartorius flat bed membrane filters and the Filta-Max™ compressed foam depth filters. These researchers concluded that the Envirochek HV filters, when subjected to a 5 second backwash, yielded a higher recovery of both *Cryptosporidium* oocysts and *Giardia* cysts in distilled and raw surface water samples. Table 1 outlines the advantages and disadvantages of filtration methods used for detection of *Cryptosporidium* in water samples.

Density gradient concentration

To further concentrate the oocysts and cysts eluted in water from filtration, a percoll-sucrose gradient method

Table 1 | Comparison of the advantages and disadvantages of isolation methods for *Cryptosporidium* from water samples

***Cryptosporidium* detection methods**	**Outline of method**	**Advantages**	**Disadvantages**
1 Pleated membrane filter			
Envirochek ™ (Simmons *et al.* 2001; McCuin & Clancy 2003)	Pleated filter within a capsule (6 cm × 21 cm). The hydrophilic polyethersulfone membrane, pore size of 1 μm, surface area 1,300 cm^2.	Increased surface area enables easier filtration of large volumes of water.	Major capital outlay required. Filter is not reusable.
Envirochek HV ™ (Simmons *et al.* 2001; McCuin & Clancy 2003)	As above; polyester, hydrophilic membrane.	No contact with filtration material; minimizes risk of exposure or cross contamination. Ability to back flush filter improves recovery.	
2 Flat-bed membrane filtration			
Millipore – mixed cellulose ester membrane filter (cellulose acetate/cellulose nitrate) (Hsu *et al.* 2001)	142 mm in diameter with a pore size of 3 μm.	Simple method with minimal capital outlay.	High turbidity sample difficult to filter. Several membranes may be required.
Sartorius – cellulose acetate membrane filters (Hsu *et al.* 2001)	142 mm in diameter with a pore size of 1.2 μm.		Excessive manual handling of samples. Potential exposure risk. Potential risk of cross-contamination.
3 Compressed foam depth filtration			
Filta-Max ™ (McCuin & Clancy 2003)	Foam depth filter held within capsule made up of several compressed foam discs that expand on elution. The surface area of the compressed discs has an equivalent pore size of 1 μm.	Relatively simple method. Automation of wash procedure available. Reproducible results.	Major capital outlay required. Excessive manual handling of samples. Potential exposure risk. Filter is not reusable. Potential risk of cross-contamination from reusable capsule.

with a specific gravity of 1.09 to 1.10 has been used in many laboratories (Dubey *et al.* 1990; Vesey *et al.* 1995; Kilani & Sekla 1997). The concentrated sample obtained from centrifuging the filtrate is layered onto the gradient. After centrifugation, the layer containing the oocysts and cysts is removed, leaving behind most of the debris. Oocysts/cysts in environmental samples may also be attached or compacted to other particles, which may significantly decrease their recovery using this concentration method.

Immunomagnetic separation concentration

Immunomagnetic separation (IMS) is alternatively used to further concentrate the oocysts and cysts eluted in water after filtration. Antibody coated magnetic particles are used to capture and selectively separate oocysts and cysts from debris in solution by using a target-specific antibody bound to a magnetic particle (immunobead). After sample contact with the beads, a magnet is applied to the container, removing the beads to the side of the tube. The supernatant is then discarded, leaving the oocysts/cysts attached to the beads. The beads can then be detached from the oocysts and cysts by an acid dissociation step, leaving the oocysts/cysts remaining in solution. This procedure also reduces the concentration of substances that may interfere with further testing of the sample (Rochelle *et al.* 1999) (Figure 1).

Immunofluorescent staining

The oocysts and cysts are identified and enumerated microscopically by staining the oocysts and cysts with a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody. Monoclonal antibodies can be developed from either immunoglobulin M (IgM) or IgG3 derived from mice serum (Weir *et al.* 2000). There have been reported incidences of non-specific binding using these monoclonal antibodies (Vesey *et al.* 1997*a*). However, Weir *et al.* (2000) developed a highly specific IgG_1 monoclonal antibody specific to *Cryptosporidium* oocyst and *Giardia* cyst wall antigens which produces fewer incidences of non-specific binding with debris in water samples.

These antibodies, once attached to the oocyst/cyst wall, fluoresce a bright green when viewed using the appropriate filter cubes (wave length 450–590 nm, FT 510, LP 515) of an epifluorescent microscope.

Immunofluorescent staining of oocysts does not provide information on viability or whether the species detected is of public health significance. Therefore, further testing is required if these concerns need to be addressed. It must also be noted that the antigen on the oocyst/cyst walls, which is recognized by all commercially available antibodies, is slowly shed in the environment. Although this antigen has no effect on viability, it can also be removed by oxidizing agents, such as chlorine, therefore viable oocysts/cysts which have been treated with high levels of chlorine may

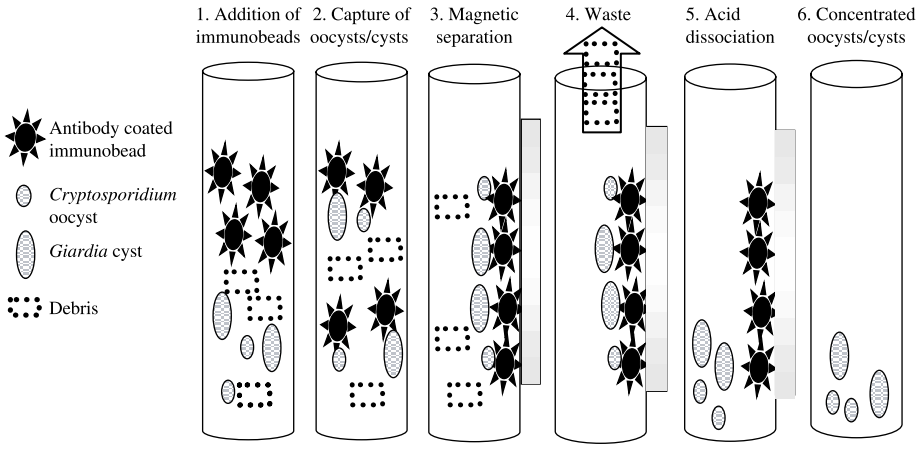

Figure 1 | The six primary steps in immunomagnetic separation of *Cryptosporidum*: 1) addition of immunobeads, 2) capture of oocysts and cysts by beads, 3) magnetic separation of immunocomplex formed from debris, 4) discarding of waste, 5) acid dissociation of bead immunocomplex and 6) concentration of oocysts/cysts left in tube.

not be detectable using the FITC-labelled antibodies (Moore *et al.* 1998).

Viability determination

Several methods to determine oocyst viability have been developed over the years. These have included cell culture (Newman 1995), animal infectivity assays using neonatal mice (Bukhari *et al.* 2000), vital dye staining (Campbell *et al.* 1992), fluorescence *in situ* hybridization (FISH) (Vesey *et al.* 1998), *in vitro* excystation (Vesey *et al.* 1997*b*) and PCR (Morgan & Thompson 1998). Some of these methods, such as *in vitro* excystation, are considered more appropriate to assess the efficacy of disinfection processes, where high numbers of oocysts can be examined. Others, such as the FISH method, are appropriate when investigating a suspected contaminated water supply or swimming pool, as the result can be obtained in a short period of time.

Vital dye staining

Vital dye staining using 4', 6'-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) has been claimed to be a rapid method for determining oocyst viability (Campbell *et al.* 1992). The precision of this method, however, has been questioned as the method is based on detecting the activity of DNA by the exclusion or inclusion of two fluorogenic vital dyes, DAPI and PI (Campbell *et al.* 1992). It has been argued that oocysts that show positive results with DAPI but are negative with PI are as viable as oocysts with negative DAPI and negative PI. Furthermore, it has been shown that measuring the activity of DNA does not always indicate viability as some residual activity may remain long after the cell has been dead. It has also been shown that PI cannot enter an intact cell wall and only cells with disrupted or broken walls are stained with PI (Belosevic *et al.* 1997).

Jenkins *et al.* (1997) demonstrated that fresh oocysts from a faecal sample were impermeable to DAPI and that the temperature has a direct impact on the permeability of the oocysts. Campbell *et al.* (1994) suggested that oocyst permeability should be considered when this viability procedure is used for environmental and disinfection studies. They also suggested that a pre-incubation step in Hanks-balanced salt solution at 37°C would improve the oocysts' permeability to DAPI.

Jenkins *et al.* (1997) also performed a comparison between the DAPI/PI assay with the *in vitro* excystation method using formaldehyde-fixed oocysts and cysts treated with sodium hypochlorite. The results of the DAPI permeability assays were the same for treated and untreated oocysts, while the excystation experiment showed no excystation in the 'killed' oocysts. This demonstrated that oocyst wall permeability was the basis of the vital dye assay rather than viability of the cell itself (Robertson *et al.* 1992; Belosevic *et al.* 1997; Jenkins *et al.* 1997). Despite this, DAPI can still be used to visualize the internal morphology of the oocysts, to detect the presence or absence of sporozoites, thus aiding in the identification of the organism. Depending on the permeability of the oocysts to the stain, the internal morphology of the organisms (i.e. the nuclei of the sporozoites) will fluoresce blue.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a viability technique that uses a fluorescently labelled (Texas Red) oligonucleotide probe (5' CGG TTA TCC ATG TAA GTA AAG 3') to target a specific sequence of 18S rRNA in the 128–148bp region of *C. parvum*. Ribosomal RNA is believed to degrade rapidly after cell death (Vesey *et al.* 1998). Therefore, FISH can determine both viability and speciation of oocysts that fluoresce or contain fluorescent sporozoites. Oocysts that do not fluoresce are considered non-viable. Concentrated samples are incubated with the probe and, coupled with FITC staining, this procedure allows for detection and enumeration of oocysts and viability determination in the single sample preparation procedure. The entire procedure including sample concentration takes approximately 3 h and is useful when rapid results are required in an outbreak investigation (Vesey *et al.* 1998).

Excystation

This method replicates the environment in which the oocysts progress onto the next stage of the life cycle. Excystation is the action by which the sporozoites are released from the oocysts when the suture in the cell wall is dissolved (Figure 2).

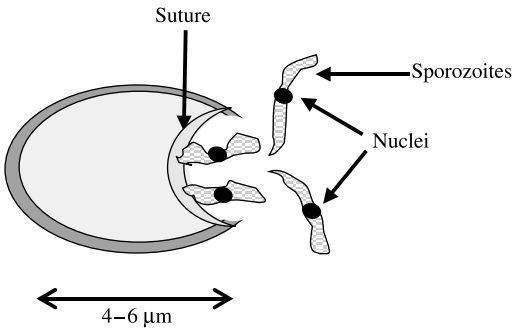

Figure 2 | Sporozoites exiting the oocysts through the suture opening. Sporozoites and nuclei fluoresce blue with the DAPI stain.

The excystation procedure also improves the permeability of oocysts to DAPI staining, allowing for effective visualization of the internal morphology of oocysts (Campbell *et al.* 1992). Comparative studies have also shown that excystation may overestimate oocyst viability (Bukhari *et al.* 2000) and that it underestimated inactivation of oocysts when using ozone as the disinfectant when compared with animal infectivity assays (Finch *et al.* 1993). High numbers of oocysts (>100) are required for this method to be effective. The number of intact and empty oocysts before and after the excystation determines the level of viability. Oocysts are viewed microscopically and results are available within approximately 6 h. It has been shown that excystation with a pre-incubation step in acidified Hanks-balanced salt solution, is a suitable substitute for animal infectivity in evaluating the inactivation efficacy of disinfectants (Vesey *et al.* 1997*b*; Hirata *et al.* 2001).

PCR

PCR techniques have been used in conjunction with conventional concentration methods, including filtration, IMS, excystation and cell culture, and can be used to determine viability, speciation and genotype of oocysts. Primers have been developed to differentiate between human and bovine genotypes. The human genotype (now known as *C. hominis*) appears to be responsible for human infections only, whereas the bovine genotype (now known as *C. parvum*) has been found in humans, cattle, dogs, pigs, kangaroos and other mammals (Morgan *et al.* 1998; Carey *et al.* 2004). Viability can be determined by the detection of mRNA from a *C. parvum* heat-shock protein (hsp-70) gene using RT-PCR (Morgan & Thompson 1998). IMS has been shown to improve the sensitivity of the PCR assays (Hallier-Soulier & Guillot 2000). Although it is possible to detect a single oocyst by nested PCR-restricted fragment length polymorphism primers, the sensitivity of PCR is dependent on sufficient oocyst numbers being present in the sample (Sturbaum *et al.* 2001). The efficiency of the concentration procedure of the initial water sample and optimization of the DNA extraction method also determine the sensitivity of the PCR methods (Nichols & Smith 2004). Hashimoto *et al.* (2006) describe a method for genotyping individual *Cryptosporidium* oocysts obtained from sewage samples using a semi-nested PCR for the 18S rRNA gene and direct sequencing of the PCR products. Their results demonstrated that semi-nested PCR and direct sequencing can be used to characterize individual *Cryptosporidium* oocysts and to reveal the distribution of *Cryptosporidium* genotypes in environmental waters.

Molecular techniques are not currently performed routinely in water analysing laboratories because sophisticated equipment and facilities are required to accurately perform these types of analysis.

INDICATOR ORGANISMS FOR *CRYPTOSPORIDIUM*

The presence of *Clostridium perfringens* has been directly linked to faecal contamination (Bisson & Cabelli 1979). It has been suggested that spore forming *C. perfringens* be used as a surrogate indicator when assessing disinfection methods designed to inactivate *Cryptosporidium*. This is due to the resistance of the *Clostridium* spores to chlorine (Payment & Franco 1993). With the current drought situation in many countries and possible water restrictions in effect, the future may demand the reuse of recycled sewage effluent into quality water suitable for irrigation or indirect potable use. This requires a designated log reduction in organisms from the raw sewage to fully treated effluent. For example, for Class A + recycled water (treated to a level suitable for dual reticulation for households, toilet flushing, garden irrigation, washing of cars and irrigation of ready to eat crops), a 6 log reduction is required for viruses, a 5 log reduction for bacteria and a 5 log reduction for protozoan parasites (Queensland Water Recycling Guidelines 2005). To achieve such a reduction, it is important to have a suitable indicator organism for *Cryptosporidium*, as oocysts may not be present in the primary effluent in high numbers (Wohlsen *et al.* 2006*c*).

OUTBREAKS OF *CRYPTOSPORIDIUM*

Food borne outbreaks

There have been many food borne outbreaks of cryptosporidiosis. In particular, in 1995 15 out of 26 persons became ill after attending a function in Minnesota where the hostess prepared a chicken salad. The hostess operated a licensed day-care home and was reported changing nappies before preparing the salads, although she washed her hands first. The salad was moist and required extensive handling and was stored in the fridge until serving, a condition which is suitable for the survival of *Cryptosporidium* (Besser-Wiek *et al.* 1996). Rose & Slifko (1999) examined the effect of hand washing on *Giardia* cysts. When 10,000 *Giardia* cysts were added to the palm of a hand, 100 cysts remained after washing hands with soap. It is assumed that a similar finding would be demonstrated if oocysts were used. This study showed that it was still possible to contaminate foods after hand washing and reinforces the recommendation that food handlers should wash hands and wear gloves while preparing food.

Cryptosporidium oocysts have also been detected on the surface of fruits and vegetables and in foods prepared with contaminated water. There have been several unpasteurized apple cider outbreaks in the United States. These have been caused by factors ranging from fruit collected from the ground of a farm where a calf was infected with *Cryptosporidium* (Millard *et al.* 1994), to fruit washed with well water located near a dairy farm (Anon 1997). The consumption of unpasteurized cow's milk poses a major risk of infection due to the frequent occurrence of parasites on dairy farms (Garber *et al.* 1994). For example, a cryptosporidiosis outbreak due to the consumption of unpasteurized milk occurred in Queensland, Australia, in 2001. Eight children were diagnosed with cryptosporidiosis and four were hospitalized. Investigations by the regional public health unit into the potential source of infection found strong evidence to implicate the consumption of unpasteurized milk in the two weeks prior to the onset of illness as the most likely source of infection (Harper *et al.* 2002).

Water borne outbreaks

Water borne outbreaks caused by *Cryptosporidium* are a major concern for public health. There have been many water borne outbreaks reported worldwide (Thwaites 1997; Craun *et al.* 1998; Morris *et al.* 1998; Clancy & Hansen 1999). There have also been many reported outbreaks due to contaminated recreational (Kebabjia 2001; Puech *et al.* 2001) and drinking water supplies (MacKenzie *et al.* 1994; Thwaites 1997).

It was determined that the cause of the Milwaukee outbreak in 1993 was poor operation of the water treatment plant, which became overloaded with dirty water after an unusually large spring thaw into Lake Michigan. This lake, which serves as the main water supply for the city, received animal and human waste with the run-off (Thwaites 1997).

The most significant Australian incident, the 'Sydney Water Crisis', occurred in 1998. In July, August and September, high counts ($>1{,}000\ 100\,l^{-1}$) of *Cryptosporidium* and *Giardia* were detected in the treated water supply (Mazounie *et al.* 2000). The 3.5 million consumers (1.5 million properties) in the Sydney region were issued with boil water alerts on the three occasions, lasting from 8 to 14 days' duration. After the first incident, an independent government inquiry was initiated. More than 12,000 businesses and individuals sought compensation from the water authority (i.e. Sydney Water). Consumer confidence and trust in the water authority was diminished, the water authority lost several key staff members, and the facility was restructured and placed under ministerial control. The cost of the crisis was estimated at AUS$33 million, including customer rebates, lost revenue, staff, testing costs and damages claims (Stein 2000). The inquiry determined that there were several potential sources of contamination in the catchment, including the contribution from extreme rainfall following a period of drought and an operational failure at the water treatment plant. Although no incidents of illness were reported, the issuing of boil water alerts was deemed the appropriate response to the incident to protect the health of the population (Stein 2000).

INACTIVATION OF OOCYSTS

The most commonly employed methods to inactivate or remove *Cryptosporidium* are heat treatment (Fayer 1994), ozonation (Peeters *et al.* 1989), UV sterilization (Dykson *et al.* 1998), chemical disinfection (Carpenter *et al.* 1999) and filtration (Parker & Smith 1993). These methods are briefly described below.

Heat

It has been shown that high temperature, short time pasteurization (Harp *et al.* 1996) is sufficient to destroy the *Cryptosporidium* oocysts in water and milk. According to these studies, oocysts could be inactivated when water temperature was kept at 72.4°C or above for one minute or when it was kept at 64.2°C or above for two minutes. Commercial pasteurization uses 71.7°C for 15 seconds, which is sufficient to inactivate *Cryptosporidium* oocysts (Fayer 1994; Harp *et al.* 1996).

Ozone

Ozone has been shown to effectively inactivate *Cryptosporidium* in water. Ozone is a powerful disinfectant produced by passing an electrical current through air to produce ozone gas (O_3) and is commonly used to disinfect drinking and wastewaters (Peeters *et al.* 1989). *Cryptosporidium* oocysts are 30 times more resistant to ozone than are *Giardia* cysts (Korich *et al.* 1990). Ozone has higher levels of disinfection than chlorine or UV because of its powerful biocidal oxidising properties and, in addition to reacting with pathogens, it reacts with other constituents commonly present in natural waters. For instance, it has been shown that an ozone concentration of 1–2 mg l^{-1} for 5 min is required to inactivate 99% of oocysts compared with chlorine concentration of 80 mg l^{-1} for 90 min (Korich *et al.* 1990). Because of this powerful activity, ozone is often used as the last line of defence in water treatment facilities to inactivate *Cryptosporidium*.

Wohlsen *et al.* (2007) assessed the effectiveness of ozonated water drawn from a working water treatment plant to inactivate a concentration of 1,000 oocysts per litre. Despite rapid degradation of ozone in the sample containers, there was up to 92% inactivation of oocysts in summer and 92.8% in winter temperatures.

Ultraviolet

UV radiation has been demonstrated to effectively inactivate *Cryptosporidium* in drinking water. It does not involve the addition of a chemical to the water and is an effective germicide (Dykson *et al.* 1998). UV doses typically used in water and wastewater treatment plants (30–40 mJ cm^{-2}) have been shown to effectively inactivate oocysts (Shin *et al.* 2001). Although temperature, correct operation and contact time of the light source with the water is critical in assessing the efficacy of UV irradiation, it has been shown that UV doses of 230 mWs cm^{-2} produced a 2-log reduction in oocyst viability (Morita *et al.* 2002). Despite *Cryptosporidium* possessing the gene which enables oocyst repair after exposure to UV light (Rochelle *et al.* 2005), there has been some evidence to demonstrate that *Cryptosporidium* is unable to repair itself under typical treatment plant conditions (Shin *et al.* 2001; Morita *et al.* 2002; Rochelle *et al.* 2005).

Chlorine

Chlorine has been traditionally used for disinfection of drinking water at concentrations of 1–3 ppm (NHMRC 2004). However, the standard chlorine disinfection procedure at this concentration is not effective enough to inactivate *Cryptosporidium* oocysts (Korich *et al.* 1990). The presence of faecal material in water can also have a negative effect on the efficacy of chlorine to inactivate oocysts resulting in higher levels of chlorine required for disinfection (Carpenter *et al.* 1999).

Excystation experiments indicate that a sodium hypochlorite concentration of 3 ppm for 7 days is required to significantly reduce the percentage of viable oocysts while 80 ppm of free chlorine is needed to produce 0% of excystation after 2 h (Korich *et al.* 1990). Therefore, in a swimming pool, 80 ppm of chlorine is recommended to treat the pool after a faecal accident or after a known contamination from *Cryptosporidium* oocysts has occurred (Queensland Health Swimming and Spa Pool Water Quality and Operational Guidelines 2004).

Environmental oocysts may not necessarily be more easily inactivated than freshly shed oocysts. Studies have shown that oocysts in surface waters are just as resistant to inactivation by chlorine and monochloramine as control oocysts that were kept in the laboratory fridge at 4°C (Chauret *et al.* 1998).

Filtration

A study was performed where *C. parvum* oocysts were shaken with sand at intervals ranging from 5 minutes to

2 hours. Increasing the duration of shaking caused oocysts to lose their contents and fragment. Following agitation with sand, chlorination increased the number of non-viable oocysts. This data suggested that the abrasion of oocysts during their passage through rapid sand filters may increase the effectiveness of chlorination (Parker & Smith 1993). Studies have also shown that diatomaceous earth filtration has the ability to remove >6 logs of *Cryptosporidium* oocysts from water. Removal of turbidity and total coliform bacteria was dependent on the grade of diatomaceous earth (Lange *et al.* 1986; Ongerth & Hutton 1997).

CONCLUDING REMARKS

Accurate detection and enumeration of *Cryptosporidium* oocysts in drinking and/or recreational waters has been a major concern for water authorities. With water supplies diminishing because of the current drought conditions in many parts of the world and the increasing population of city regions, the quality of the water supply has never been more important. To maintain the health and welfare of water consumers and to ensure public confidence in the water authorities, vigilant monitoring of source and treated water supplies must be undertaken. Part of the requirements for achieving optimal water quality necessitates the use of highly sensitive, reproducible and reliable methods for detection of pathogens such as *Cryptosporidium* and *Giardia*.

It has been a common belief that oocysts should be present in surface water samples after rainfall mainly because animal faeces will be washed into the surface waters. For this reason, many studies have assessed the abundance of *Cryptosporidium* in catchment areas. The fact that each catchment has unique land-use characteristics makes it rather difficult to fully apply conclusions obtained by other studies regarding the sources and persistence of the *Cryptosporidium* contamination from one catchment to another, as the prevalence of oocysts in one area may not be equivalent to those of another.

Many methods are used to detect *Cryptosporidium* in surface water. While these methods require the concentration of a large volume of water sample, they are all technically demanding and require significant equipment resources. An incorrect report on a water sample due to the inability of the method to detect a low level of oocysts may have health and legal implications. The problem common to all of the current detection methods is that no method can detect 100% of oocysts present in a sample. Recent studies have indicated that modification of the existing methods can greatly improve the detection rate of these pathogens in water samples. However, there is still much demand to further improve the recovery efficiency of these methods and to develop rapid and simple molecular techniques not only to speciate the oocysts (i.e. *C. hominis* specific to humans and *C. parvum*, present in humans and cattle) but also to be able to quantify the number of these pathogens in surface waters.

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