Development and Application of a Molecular Viability Assay for *Cryptosporidium parvum* Based on Heat Shock Protein 70 Gene Expression

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ABSTRACT

The aquatic protozoan pathogen *Cryptosporidium parvum* causes serious problems in water treatment plants. Its small size allows it to evade most physical removal barriers, becoming a major cause of diarrhoeal disease. Cryptosporidiosis is self-limiting in immuno-competent people, but can be life-threatening to immuno-compromised people. Multiple physical and/or chemical barriers, e.g. UV light, are used to remove or inactivate oocysts. It is important to have a reliable method to estimate numbers of viable *C. parvum* in order to monitor water supplies and evaluate protection measures.

This study aims to establish an RT-PCR assay for viable *C. parvum* based on expression of the heat shock protein 70 (*hsp70*) gene. For the assay, oocysts are heat shocked at 45°C to increase *hsp70* mRNA (to increase assay sensitivity) and then lysed to release nucleic acids. Oligo $(dT)_{25}$ Dynabeads® are used to isolate mRNA and a DNaseI step removes genomic DNA. The RT-PCR step converts mRNA to cDNA and this product is analysed by electrophoresis on an agarose gel.

Many studies have investigated UV as a means to inactivate oocysts, but none so far have used an RT-PCR viability assay. UV inactivation may take two forms: oocysts can either be metabolically inactive or they can remain metabolically active but are rendered non-infective. First, the cidal UV dose for *C. parvum* oocysts was determined and was found to be 1100 mJ/cm^2; this value is much higher than previously reported. Next, photoreactivation of UV-killed oocysts was investigated and found not to occur in *C. parvum*, which is inline with other observations. But, contrary to previous studies, trials reported here showed that *hsp70* mRNA is still detectable inside heat inactivated oocysts even after 70 hours at room temperature. Other experiments conducted include trials using β-tubulin gene as a viability marker and studying the effect of *hsp70* mRNA stability on heat inactivation of oocysts. Using the β-tubulin gene as a genetic marker in the RT-PCR assay found a cidal dose of only 10 mJ/cm^2 was required for *C. parvum* oocysts, which corroborates previous work and can be explained by β-tubulin mRNA being less stable than *hsp70* mRNA.

The RT-PCR oocyst viability assay developed here is both efficient and effective. Overall the results presented show the importance of a reliable viability testing method and also that the best choice of gene target depends on the method of oocyst inactivation. The high apparent stability of *hsp70* mRNA within heat inactivated oocysts limits the utility of this gene target and this phenomenon warrants further investigation. Future applications of the general RT-PCR oocyst viability assay method include development into a high throughput system for routine determinations of viable oocyst numbers in contaminated water. Other gene loci can also be examined as alternative viability markers now that the complete *C. parvum* genome sequence is available.

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	base pairs (DNA)
cDNA	synthetic DNA, generated from RNA
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate (dATP, dTTP, dGTP, dCTP)
EDTA	ethylene diamine tetra-acetic acid
FITC	fluorescein isothiocyanate
hsp70	heat shock protein 70
IMS	Immunomagnetic separation
Kb	kilobases (DNA)
mJ/cm^2	millijoule per square centimetre
mRNA	messenger ribonucleic acid
NIST	National Institute of Standards and Technology
nm	nanometre
PBS	phosphate buffered saline
RFLP-PCR	Restriction Fragment Length Polymorphism-PCR
RNase	ribonuclease
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
TAE	tris acetate EDTA buffer
TBE	tris borate EDTA
TE	tris-EDTA buffer
USEPA	United States Environmental Protection Agency
UV	ultraviolet light
V	volts

CHAPTER 1 : INTRODUCTION

1.1 Overview

The protozoa *Cryptosporidium parvum* is a major cause of diarrhoeal disease in humans (Quintero-Betancourt *et al.*, 2002; Rose *et al.*, 2002). The oocyst stage (Figure 1.1), excreted in faeces of infected humans and animals, has been responsible for recent waterborne outbreaks of human cryptosporidiosis (Wagner-Wiening and Kimmig, 1995). Cryptosporidiosis is self-limiting in immuno-competent people, but can be life-threatening to immuno-compromised people (Martins and Guerrant, 1995; Morgan and Thompson, 1998).

There are various methods to determine viability of *C. parvum* (Section 1.10), but all have limitations such as, non-specificity and low sensitivity, labour intensive and expensive. To avoid these problems a reverse transcription polymerase chain reaction (RT-PCR) assay using the expression of a heat shock protein *(hsp70)* gene can be employed. Briefly, the method for the assay is as follows; oocysts are captured using immunomagnetic separation (IMS) from the sample suspension and heat shocked at 45°C for 12 minutes. These whole oocysts are then lysed to release nucleic acid. Messenger RNA (mRNA) is captured using IMS and the sample then undergoes a DNaseI treatment followed by a one-step RT-PCR using specific primers for the *C. parvum hsp70* gene. The presence of the 361bp *hsp70* product visualised in an agarose gel indicates viability of the sample.

It is an advantageous method because of its sensitivity, as the *hsp70* mRNA is expressed at increased levels when under heat shock stress and can therefore detect small numbers of viable oocysts (Gobet and Toze, 2001; Morgan and Thompson, 1998). Expression of the *hsp70* mRNA is only found in viable oocysts and has a short half-life (Stinear *et al.*, 1996); these are beneficial properties of a viability marker.

Oocysts are small and often pass through physical water treatment processes (Hargy *et al.*, 2000). Consequently irradiation with ultraviolet (UV) light has grown in popularity as a disinfecting technique since *Cryptosporidium* is highly chlorine resistant (Korich *et*

al., 1990). UV inactivates *Cryptosporidium* through formation of pyrimidine dimers between two pyrimidine bases on the same DNA strand (Lindenauer and Darby, 1994). These prevent cellular functions and inhibit the ability to replicate. However, it has been proposed that *Cryptosporidium* may have the ability to repair this damage and become viable again by a mechanism called photoreactivation (Oguma *et al.*, 2001; Shin *et al.*, 2001).

The research undertaken in this study will develop an RT-PCR oocyst viability assay so that a sensitive, specific and rapid method can be used to determine the viability of *C. parvum* oocysts. This RT-PCR assay will then be used to determine an effective cidal UV dose for oocysts and to determine the presence/absence of photoreactivation.

1.2 *Cryptosporidium* and Cryptosporidiosis

The genus *Cryptosporidium* (Tyzzer, 1907) is a eukaryote in the phylum Apicomplexa. All species of *Cryptosporidium* are obligate, intracellular (but extracytoplasmic), protozoan parasites that undergo endogenous development ending with the production of an encysted stage (oocyst) that is released in the faeces of the human or animal host (Figure 1.1). For the majority of species in the phylum the oocyst stage is of primary importance for the dispersal, survival and infectivity of the parasite. It is also the stage of major importance for detection and identification of the parasite (Fayer *et al.*, 2000).

Cryptosporidium parvum oocysts are 4-6 μm in size and contain four sporozoites. Both thin and thick-walled oocysts occur; the thin-walled oocysts are speculated as being responsible for endogenous infection (auto-infection) and the thick-walled oocysts hardier for transmission *via* the environment (Baeumner *et al.*, 2001). This hardiness results from the impermeability of the oocyst wall, making it resistant to agents that may stimulate late excystation or exposure to lethal chemicals in the environment (Jenkins *et al.*, 1997). The oocyst is metabolically dormant (Jenkins *et al.*, 1997) and in conjunction with the low infective dose, makes *C. parvum* a significant waterborne pathogen (Baeumner *et al.*, 2001; Stinear *et al.*, 1996). Transmission is *via* the faecal-oral route, causing a gastrointestinal illness resulting in diarrhoea, stomach cramps, nausea and sometimes anorexia (Call *et al.*, 2001; Chung *et al.*, 1999; Martins and

Guerrant, 1995). For immunocompromised individuals, diarrhoea can last for months or life since there is no effective treatment. However, paromomycin and azithromycin have been shown to be useful in improving symptoms (Martins and Guerrant, 1995). The first human case of cryptosporidiosis was reported in 1976 (Meisel *et al.*, 1976; Nime *et al.*, 1976) with *C. parvum* being the major species responsible for clinical disease in humans and domestic mammals (Baeumner *et al.*, 2001).

Antigens on the surface of the oocyst wall may stimulate an antibody response in immunised animals and these antibodies can be labelled to aid identification of oocysts. However, many oocyst wall antigens are conserved within the genus *Cryptosporidium* and appear in several species, consequently, there are currently no antibodies to reliably differentiate species (Fayer *et al.*, 2000).

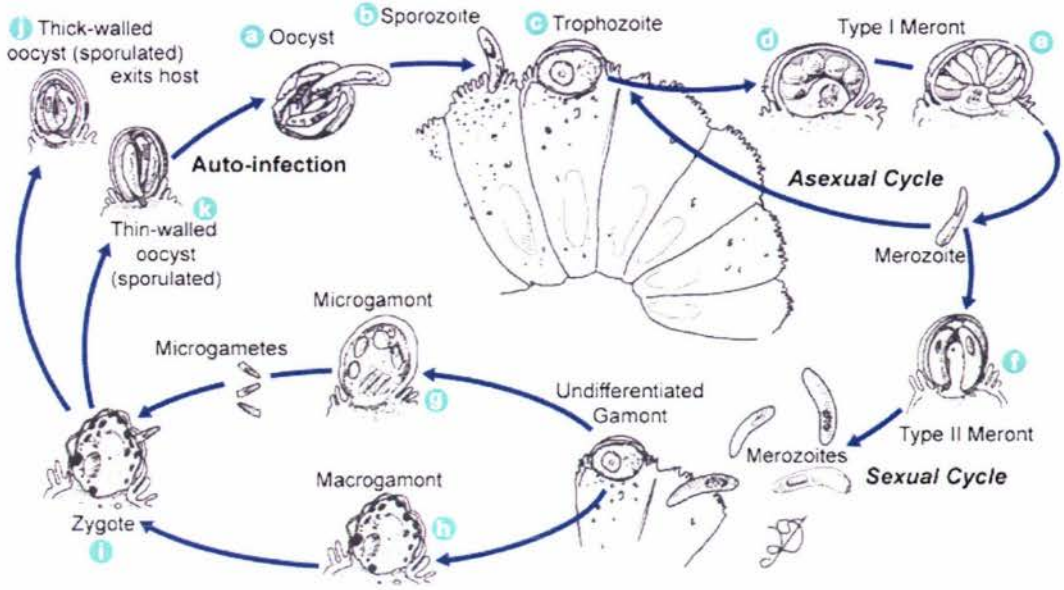

Figure 1.1: ***Cryptosporidium*** **lifecycle.** Key: **(a)** excystation of the oocyst, **(b, c)** sporozoites released and parasitise epithelial cells of gastrointestinal tract, **(d, e, f)** parasites undergo asexual multiplication (schizogony or merogony), followed by sexual multiplication (gametogony) where **(g)** microgametes (male, produced by the microgamont) fertilise the **(h)** macrogamont (female) and a **(i)** zygote is formed, which encysts (sporulates) to become **(j, k)** an oocyst, either thick- or thin-walled. Taken from: (Center for Disease Control and Prevention).

1.3 Disinfection of Water

1.3.1 Ultraviolet (UV) Light and Physical Removal of *Cryptosporidium* Oocysts

Cryptosporidium is robust in its vegetative form, the oocyst, and is highly resistant to chlorine-based disinfection (Carey *et al.*, 2004; Mackey *et al.*, 2000; Oguma *et al.*, 2001). Even after a prolonged exposure of 18 hours to free chlorine, oocysts were not inactivated (Korich *et al.*, 1990). *Cryptosporidium* is also very small and can pass through physical water treatment processes (Carey *et al.*, 2004). Downes and Blunt (1877) first noted the germicidal effects of sunlight and mercury lamps were used as disinfectant UV light sources from as early as 1901 (Downes and Blunt, 1877; United States Environmental Protection Agency *et al.*, 2003). But, it has not been until recently that interest has grown in using UV as a disinfecting agent in conjunction with physical removal methods. These removal methods for *Cryptosporidium* include coagulation, flocculation, sedimentation and filtration and are used to help prevent oocysts passing through treatment plants (Carey *et al.*, 2004; Linden *et al.*, 2001; Oguma *et al.*, 2001). Use of UV treatment for water after physical removal methods, ensures inactivation of any remaining *Cryptosporidium* oocysts and following this with chlorine provides a lasting disinfectant against bacteria (Karanis *et al.*, 1992). Bacterial biofilms and particles provide shelter from UV for *Cryptosporidium* as well as fouling the UV lamps and can be reduced by chlorine treatment as this removes nutrients the bacteria use to live (Cotton *et al.*, 2001). This "multiple-barrier" approach appears to inactivate the highest number of *Cryptosporidium* (Nwachcuku and Gerba, 2004; United States Environmental Protection Agency *et al.*, 2003). The use of UV disinfection systems is therefore beneficial for many reasons including that no additional chemicals beyond chlorine are required, no hazardous disinfection by-products (DBP) are generated, only short contact times are required (Carey *et al.*, 2004) and system maintenance is simplified (Oguma *et al.*, 2001; Rose *et al.*, 2002).

1.3.2 Water Quality

The first public drinking water disinfection installation was set up in Marseilles, France in 1910 (United States Environmental Protection Agency *et al.*, 2003). In 1929 a link

between the efficacy of UV disinfection and absorption of UV light by nucleic acids was made. However, it was not until 1955 before reliable UV installations were being used to regularly disinfect water (United States Environmental Protection Agency *et al.*, 2003). In 1996, there were over 2000 UV disinfection systems treating drinking water in Europe (United States Environmental Protection Agency *et al.*, 2003).

The Drinking Water Standards for New Zealand 2000 (Ministry of Health New Zealand, 2000) are currently being reviewed by the Ministry of Health and New Zealand Water and Wastes Association. In April 2004, UV disinfection was approved for up to 3-log removal of *Cryptosporidium* (i.e. 99.9% inactivation). This was based on United States Environmental Protection Agency (USEPA) guidelines (United States Environmental Protection Agency *et al.*, 2003), providing water had a turbidity of less than 1.0 nephelometric turbidity units (NTU) for 95% of the time and not greater than 2.0 NTU for 100% of the time. Turbidity is quantified in NTU, which are measured by determining the amount of light scattered by particulate material in water, as this correlates well with the concentration of these particles (United States Environmental Protection Agency *et al.*, 2003). The necessary log removal level for *Cryptosporidium* is dependent on the quality of the input source water. For example, 3-log removal is required when there are less than 7.5 oocysts per 100 L source water and 5-log removal is required when 100-300 oocysts per 100 L source water are present (United States Environmental Protection Agency *et al.*, 2003). In March 2003, there were 350 drinking water supplies in New Zealand using UV for disinfection but a significant proportion of these were failing to produce water complying with NZ bacteriological standards (Taylor, 2003).

Despite the routine use of turbidity measurement as a diagnostic tool to determine pathogen presence, Cinque *et al.* (2004) found that turbidity *per se* did not directly imply a high pathogen likelihood. Therefore due to the possibility of false positive results it was concluded turbidity was not a good measure of pathogen presence (Cinque *et al.*, 2004). However, USEPA guidelines were adhered to prior to a *Cryptosporidium* outbreak in Nevada, USA demonstrating that low turbidity levels do not necessarily ensure a safe drinking water supply either (Carey *et al.*, 2004). In addition, there is no correlation between the presence of coliform indicator bacteria and *Cryptosporidium*

(Carey *et al.*, 2004) or other reliable biological indicators that indicate the potential presence or absence of oocysts.

1.4 UV Dose Determination and Effectiveness

1.4.1 UV Dose Determination

To test the effectiveness of UV light for inactivating *Cryptosporidium* in a laboratory environment, lamps are housed in a collimated beam apparatus (United States Environmental Protection Agency *et al.*, 2003). This apparatus ensures a relatively uniform UV irradiance is contained within the collimating tube until it reaches the suspension of *Cryptosporidium* (United States Environmental Protection Agency *et al.*, 2003). There are general properties for this apparatus, which should be adhered to between research groups to allow comparison of results. For example, the temperature of the lamp must be kept constant in a vented area to prevent overheating of the lamp, as this can reduce UV output. Although the collimating tubes are adjustable, a recommended minimum distance from the UV lamp to the *Cryptosporidium* suspension is six times the longest distance across the suspension surface (United States Environmental Protection Agency *et al.*, 2003). A shutter mechanism should be used to control exposure of the suspension to UV light with accurate exposure times being more than 20 seconds in duration (United States Environmental Protection Agency *et al.*, 2003). The suspension of *Cryptosporidium* should be mixed, slowly to prevent a vortex, using a stir bar and a magnetic stirrer to ensure even irradiation and to prevent survival of the oocysts due to shading from UV light (Kim and Sundin, 2001; United States Environmental Protection Agency *et al.*, 2003). The irradiance at the centre of the suspension surface should be measured using a radiometer calibrated at 254 nm before and after exposure to UV light and these values averaged. This allows a UV dose to be determined, as measurements often cannot be performed with the suspension *in situ*. The radiometer calibration should be National Institute of Standards and Technology (NIST) traceable or equivalent (United States Environmental Protection Agency *et al.*, 2003). NIST traceability requires the establishment of an unbroken chain of comparisons to stated references (National Institute of Standards and Technology, 2004). When using a collimated beam apparatus, safety precautions such as safety

goggles for the eyes and latex gloves for the hands should be used to shield any UV light which escapes the collimating tubes (United States Environmental Protection Agency *et al.*, 2003).

1.4.2 UV Light Effectiveness

Despite the fact that collimated beam apparatus experiments have been conducted in a controlled and uniform manner, results obtained by research groups have differed over the past decade. Independent replication of experiments and interpretation of data can be difficult, as the information presented by research groups does not always allow for calculation of UV doses delivered, as Clancy *et al.* (2000) noted was the case with one of the first papers to report the effect of UV light on *Cryptosporidium,* Lorenzo-Lorenzo *et al.* (1993).

There are varying opinions on whether infectivity or viability should be used in determining if UV disinfection is effective for inactivation of *Cryptosporidium.* The USEPA UV Guidance Manual (2003) recommends that only assays that measure infectivity should be used. They base this recommendation on comparison of past studies; those studies which used viability-based assays such as excystation and fluorogenic dyes needed much higher UV doses than other studies which used mouse infectivity (United States Environmental Protection Agency *et al.*, 2003). UV does not alter the membrane permeability of oocysts, affecting the basis for fluorogenic dye assays, which could account for the high UV doses found (Carey *et al.*, 2004). Therefore, to determine a lower UV dose for *Cryptosporidium* inactivation using a viability assay, testing differently such as with RT-PCR is necessary.

Studies conducted which tested for oocyst viability using mouse infectivity post UV treatment rather than *in vitro* assays, such as excystation or fluorogenic stains, resulted in UV being perceived to be more effective than previously thought (Drescher *et al.*, 2001). Also, in the past, oocyst viability was often equated with infectivity, leading to significantly higher UV doses being thought to be required to inactivate *Cryptosporidium* than in recent studies (Drescher *et al.*, 2001). For example, to demonstrate this point, Drescher *et al.* (2001) used mouse infectivity and found a 5.4-log reduction with 120 mJ/cm^2 whereas Campbell *et al.* (1995) used fluorogenic dyes

and excystation achieving only a 2- to 3-log reduction with 10 000 mJ/cm^2. Surprisingly, this is approximately a 10^4-fold difference in effectiveness. Also more recently, a significantly lower UV dose has been used to achieve a lower log reduction, but still within USEPA guidelines, by both Clancy *et al.* (2000) and Shin *et al.* (2001) (Table 1.1).

1.4.2.1 UV Light Susceptibility Factors

Clancy *et al.* (2004) investigated whether there was a difference in the doses required to inactivate five standard reference isolates of *Cryptosporidium* (Iowa, TAMU, Moredun, Maine, Glasgow) and found that 10 mJ/cm^2 was an effective dose to achieve at least a 4-log inactivation in each. The five *Cryptosporidium parvum* strains that were studied were all of *C. parvum* bovine genotype (i.e. capable of infection in both humans and animals) (Clancy *et al.*, 2004); therefore it would be of interest to determine UV susceptibility of *C. hominis* strains (i.e. capable of infection in humans only). Lower doses (2, 4 and 5 mJ/cm^2) were also effective in Iowa, Maine and Glasgow strains, demonstrating an unequal UV sensitivity between the five strains. After statistical analysis of nine UV inactivation studies, including Clancy *et al.* (2000, 2004), Craik *et al.* (2001) and Shin *et al.* (2001), the USEPA set a dose of 12 mJ/cm^2 as a benchmark to achieve a 3-log inactivation of *Cryptosporidium* 90% of the time when using a collimated beam apparatus (United States Environmental Protection Agency *et al.*, 2003).

Older oocysts have been shown to require a lower dose of UV to inactivate them than fresh ones do (Hargy *et al.*, 2000). For example a 2-log inactivation was demonstrated using mice infectivity when using 40 mJ/cm^2 for fresh and 14 mJ/cm^2 for aged oocysts respectively (Clancy, 1998). This reduction in UV dose required for inactivation could be advantageous to water treatment plants that have longer holding times prior to UV treatment.

1.4.2.2 Biodosimeters and Surrogates

Differences in UV doses required for *Cryptosporidium* inactivation are often seen between bench-scale experiments using a collimated beam apparatus and demonstration scale experiments using UV reactors (Bukhari *et al.*, 1999). However, as Bukhari *et al.* (1999) found, when the oocyst inactivation data were calculated as the log of the

viability ratio (defined as the ratio of the viability of the UV-treated oocysts to that of the process control) and plotted this versus the UV dosage, for both the demonstration and bench scale studies, they indicated that the UV dosages applied were similar for each study (Bukhari *et al.*, 1999). This shows that it is reasonable to extrapolate results of the bench-scale studies to the demonstration scale.

Bioassays using other microorganisms can be performed as another way to help determine the amount of irradiance actually reaching *Cryptosporidium* oocysts and hence the true UV dose required for inactivation. However, conflicting evidence over whether or not surrogate organisms (biodosimeters) can be used reliably has been published in recent years (Mackey *et al.*, 2002). These surrogates are often MS2 coliphage or *Bacillus subtilis* spores where log inactivation curves are obtained from measurements taken using the same UV equipment or reactor as in *Cryptosporidium* trials (United States Environmental Protection Agency *et al.*, 2003). Mackey *et al.* (2000, 2002) showed that the MS2 coliphage surrogate was correlated with *Cryptosporidium* response to UV irradiation. However, it was found that MS2 coliphage was a conservative surrogate and the conclusion drawn that a surrogate with a more comparable UV dose response would be more beneficial (Mackey *et al.*, 2002). Mofidi *et al.* (2001) showed that *Escherichia coli* could be used as a surrogate for assessing *C. parvum* disinfection by UV irradiance, although more trials were necessary to confirm how comparable the UV dose responses were.

Viruses generally require a higher UV dose to kill them than *Cryptosporidium* does with only 2-log inactivation of most viruses achieved with 140 mJ/cm^2 (Bukhari *et al.*, 1999). Bacteria also require higher UV killing doses than *Cryptosporidium* but still lower than for most viruses (Cotton *et al.*, 2001; United States Environmental Protection Agency *et al.*, 2003). Adenoviruses are double-stranded DNA viruses that are highly resistant to UV disinfection (Nwachcuku and Gerba, 2004). It is believed this resistance is due to enzymes within the host cell which are able to repair damaged sections of the double-stranded viral genome using the nondamaged strand as a template (Nwachcuku and Gerba, 2004). This could explain why viruses with single-stranded genomes are more susceptible to UV disinfection and also demonstrates the need to carefully choose a surrogate organism so as to avoid differences in UV dose-response between the surrogate and *Cryptosporidium*.

Table 1.1: **UV inactivation values for *Cryptosporidium***

Author	Inactivation testing method	UV dose used (mJ/cm^2)	Log reduction
Campbell *et al.*, (1995)	Fluorogenic dyes & excystation	10 000	2-3
Bukhari *et al.*, (1999)	Mice infectivity	41	4
Clancy *et al.*, (2000)	Mice infectivity	6	3
Drescher *et al.*, (2001)	Mice infectivity	120	5.4
Shin *et al.*, (2001)	Cell culture & mice infectivity	5	2.9-3.3

1.5 UV Light Properties

The UV-C region of the UV spectrum (which is part of the electromagnetic spectrum) has a germicidal range at approximately 200-300 nm with a peak germicidal effectiveness around 260 nm, the wavelength at which UV energy is absorbed by DNA and RNA (International Light, 1998; United States Environmental Protection Agency *et al.*, 2003). In most cases, UV light is generated by applying a high voltage across a gas mixture, which results in a continual discharge of photons. Most UV lamps used in water treatment use a gas mixture containing mercury vapour (United States Environmental Protection Agency *et al.*, 2003). UV light of different wavelengths can be produced depending on the pressure and temperature of this mercury vapour. For example, when mercury is at low vapour pressure (near vacuum; 0.001-0.01 torr) and moderate temperature (40°C), 98 percent of the UV light produced is monochromatic at 253.7 nm (Bolton, 2004; United States Environmental Protection Agency *et al.*, 2003). At higher pressures (100-10 000 torr) and higher temperatures (600-900°C), UV light is

produced over a broad spectrum in the 200-300 nm region (polychromatic) (United States Environmental Protection Agency *et al.*, 2003). This results in medium-pressure lamps having about half the germicidal efficiency compared with low-pressure lamps. However, both low-pressure and medium-pressure mercury UV lamps are used to disinfect water (Bolton, 2004) and Clancy *et al.* (2000), found that low and medium-pressure UV lamps both achieved similar levels of inactivation under collimated beam apparatus conditions.

UV dose is calculated by multiplying the average UV intensity (milliwatts per square centimetre, mW/cm^2) by the exposure time (seconds, s) and is measured in millijoules per square centimetre, mJ/cm^2 (1 mJ/cm^2 = 1 mWs/cm^2) (United States Environmental Protection Agency *et al.*, 2003) (Appendix 4.2). The UV dose being delivered to oocysts can be plotted against the level of oocyst inactivation to determine a dose-response relationship (United States Environmental Protection Agency *et al.*, 2003). Factors such as the depth and UV absorbance of the water layer, reflection and refraction off the water surface and the irradiance emitted by the lamps (temperature dependent) can affect the average intensity of UV actually delivered (United States Environmental Protection Agency *et al.*, 2003). These factors diminish the effectiveness of UV light and result in a lower dose reaching the oocysts (Taylor, 2003). Other factors include lamp-aging, biofouling of lamps and unexpected changes in water quality such as high turbidity (Cotton *et al.*, 2001; Goin *et al.*, 2004). Turbidity is caused by particles in the water, which absorb UV light and lamp fouling reduces the level of UV transmitted beyond the quartz lamp sleeve (Cotton *et al.*, 2001). The flow rate of the water can also have an effect on UV dose per oocyst, as the length of time that the oocyst receives irradiation will be reduced at a high flow rate (United States Environmental Protection Agency *et al.*, 2003). However, these concerns are less applicable at the bench-scale level when using collimated beam apparatus with a static sample compared with in a UV reactor. Therefore, when UV is to be used practically in a treatment plant these factors must be minimised for an effective dose to be provided at all times.

1.6 UV Induced Cellular Damage

The most common form of damage that ultraviolet light can cause to DNA and RNA is through absorption by adjacent pyrimidine bases (thymine or cytosine in DNA and uracil or cytosine in RNA) on the same DNA or RNA strand, and results in dimer formation (Figure 1.2) (Cotton *et al.*, 2001; Lindenauer and Darby, 1994; United States Environmental Protection Agency *et al.*, 2003). This damage causes disruption of cellular processes such as replication and transcription (Bolton and Linden, 2002; Lorenzo-Lorenzo *et al.*, 1993), resulting in inactivation of *Cryptosporidium* (United States Environmental Protection Agency *et al.*, 2003). This means microorganisms that have thymine/uracil rich DNA/RNA are more prone to damage by UV light (United States Environmental Protection Agency *et al.*, 2003). Pyrimidine (6–4) pyrimidine photoproducts are also a major class of UV damage (United States Environmental Protection Agency *et al.*, 2003). These forms of damage are unlike the inactivation mechanism that occurs with chemical disinfectants such as chlorine where inactivation occurs due to destruction of or damage to cellular structures, interfering with metabolism and hindering biosynthesis and growth (United States Environmental Protection Agency *et al.*, 2003).

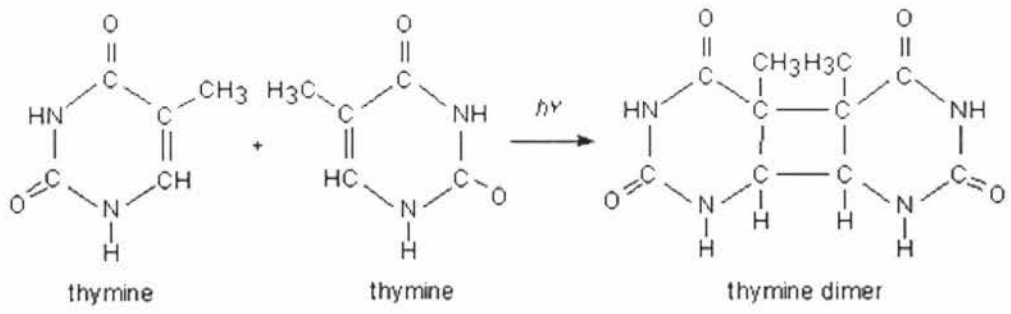

Figure 1.2: Photochemical dimerisation of two thymine bases to form a thymine dimer (taken from Bolton (2000) (Bolton, 2000))

1.7 Photo Repair and Photoreactivation

When microorganisms are exposed to UV light they are still able to retain some metabolic functions, which can enable them to repair damage done by UV light and regain infectivity (United States Environmental Protection Agency *et al.*, 2003). In 1949, Albert Kelner discovered that damage caused by ultraviolet light, such as dimer formation, could be reversed by illumination with longer wavelengths of light (Kelner, 1949). He observed a $4x10^5$-fold increase in the number of survivors of UV-irradiated *Streptomyces griseus* when exposed to 365 nm light (Kelner, 1949).

The repair of pyrimidine dimers in DNA is often referred to as photo repair. Whereas extensive repair, leading to recovery of the organism, is referred to as photoreactivation (Oguma *et al.*, 2001). Wavelengths in the near UV or violet-blue spectral range (between 310 and 490 nm) are the most effective for photo repair (Lindenauer and Darby, 1994; United States Environmental Protection Agency *et al.*, 2003). Photoreactivation can potentially counteract the effects of UV light disinfection via photo repair and reduce the efficacy of UV disinfection (Oguma *et al.*, 2001). Nucleotide excision repair (NER) is an additional repair mechanism following UV damage (Rochelle *et al.*, 2005; United States Environmental Protection Agency *et al.*, 2003).

Photoreactivation uses an enzyme called photolyase to remove UV-induced damage in DNA (Lindenauer and Darby, 1994). However, it cannot repair UV-induced damage caused during RNA synthesis and protein synthesis (Cleaver, 2003). In bacteria, the mechanism occurs in two steps; first photolyase binds to the pyrimidine dimer (Kim and Sundin, 2001) followed by a light-dependent step, which separates the dimer into two monomers and releases the repaired DNA from the photolyase. This process usually occurs in less than a millisecond. There are two different classes of photolyases: Class I are found in bacteria and lower eukaryotes and Class II in higher eukaryotes (Kim and Sundin, 2001). Factors that affect the level of photorepair achieved are the time between UV light and photoreactivating light exposure (United States Environmental Protection Agency *et al.*, 2003) and the dose of photoreactivating light. Lindenauer and Darby (1994) found the photoreactivating light dose-inactivation response to be non-

linear, as the effect of photorepair by coliform bacteria in wastewater became less pronounced as UV dose increased (Craik *et al.*, 2001; Lindenauer and Darby, 1994).

The process of photoreactivation occurs in bacteria (for example, *Escherichia coli and Legionella pneumophila*), plants and animals (Oguma *et al.*, 2001) however it is absent in placental mammals (Cleaver, 2003). Bacterioplankton are known to have both photoreactivation and dark repair mechanisms (Kim and Sundin, 2001). To ascertain the presence of photorepair mechanisms, individual organisms must be investigated since evolutionarily allied species do not necessarily have similar photoreactivation characteristics (Oguma *et al.*, 2001). All of the major genetic components of a NER complex have been identified in *C. parvum* based on similarity between *C. parvum* genomic sequences and recognised NER genes in other organisms (Rochelle *et al.*, 2005). The genes are dispersed across all eight of the *C. parvum* chromosomes with the exception of chromosome five (Rochelle *et al.*, 2005). To date, there have been no genes identified encoding photolyases in the *C. parvum* genome (Rochelle *et al.*, 2005).

1.7.1 Determination and Prevention of Photoreactivation

To establish the sensitivity of different organisms to UV, as well as quantifying the extent of DNA repair, an endonuclease sensitive site (ESS) assay can be performed. This assay determines the number of UV-induced pyrimidine dimers formed in genomic DNA following irradiation with UV light (Oguma *et al.*, 2001). Oguma *et al.* (2001) investigated the relationship between the ESS assay and animal infectivity in *C. parvum* to determine whether frequency of infectivity increased with photorepair and photoreactivation (Oguma *et al.*, 2001). They concluded that *C. parvum* could not recover by photoreactivation despite photorepair of pyrimidine dimers being observed in the genomic DNA. Shin *et al.* (2001) also found that photoreactivation did not occur in *Cryptosporidium,* as their oocyst preparations did not regain infectivity after inactivation with UV light followed by light at 365 nm (Shin *et al.*, 2001).

Avoidance of exposure to sunlight for 30 minutes immediately following UV treatment as well as following the UV process with a longer lasting disinfectant, such as chlorine, can help to prevent photoreactivation effects. A sufficiently high initial dose of UV is also recommended. However, it is unknown whether an increased UV dose or another

disinfectant would be sufficient to prevent dark repair also (United States Environmental Protection Agency *et al.*, 2003). It has been suggested that a broad spectrum of UV light, such as that produced with medium-pressure UV lamps, may be more effective at inactivating microorganisms and thereby makes it more difficult for photoreactivation to occur (Mofidi *et al.*, 2001). This is because broad wavelengths cause damage to other cellular molecules, in addition to DNA, such as proteins and membranes (Shaw and Warne, 2003).

1.8 Dark Repair

Dark repair refers to mechanisms other than photorepair, such as excision repair. As the name suggests, repair of DNA often occurs without the aid of light (Oguma *et al.*, 2001), however the term is misleading as dark repair can also occur in the presence of light. This makes its presence difficult to detect (United States Environmental Protection Agency *et al.*, 2003). Excision repair is an enzyme-mediated process where the damaged section of DNA is removed and regenerated using the existing complimentary strand of DNA (United States Environmental Protection Agency *et al.*, 2003). Both Oguma *et al.* (2001) and Shin *et al.* (2001) demonstrated that no dark repair occurred in *Cryptosporidium* when oocysts were exposed to dark conditions following UV irradiation. However, Oguma *et al.* (2001) did find repair to genomic DNA despite a lack of animal infectivity by the oocysts.

1.9 UV System Costs

In general, small water treatment systems have high total unit costs per volume of water treated (dollars/1000 gallons or dollars/3800 litres) compared with larger systems. This is partly because costs do not decrease in proportion to a reduction in flow (Cotton *et al.*, 2001). In America, a small system is considered to be one that supplies water to less than 10 000 people or has an average production rate of less than 1 million gallons (3.8 million litres) per day (United States Environmental Protection Agency *et al.*, 2003). The UV dose chosen for a treatment system has a significant effect on costs. For example, a cost analysis was performed using a UV dose of 140 mJ/cm^2 (required to inactivate viruses) and a dose of 40 mJ/cm^2 (chosen as a conservative requirement to

inactivate *Cryptosporidium*) and showed a 50% increase in capital costs with 140 mJ/cm^2 (Cotton *et al.*, 2001). It has been estimated that at 40 mJ/cm^2, one million US gallons (3.8 million litres) would cost approximately US$10 to process (Bukhari and LeChevallier, 2004). This increased cost for treatment of water at 140 mJ/cm^2 is due to increased UV equipment costs and larger building requirements (Cotton *et al.*, 2001). Equipment costs also depend on whether low or medium-pressure lamps are used. For example, low-pressure lamps are the least expensive but last twice as long (typically one year) before requiring replacement compared with medium-pressure lamps (Cotton *et al.*, 2001). However, more low-pressure lamps are required since they have a lower effective output than medium-pressure lamps which leads to an increase in capital and operational costs as bigger buildings are required (Cotton *et al.*, 2001). It is typical for UV equipment to be placed after water filtration and chemical disinfectants in the treatment system, as this prevents particles within the water reducing the UV dose received by microbes, making the system more cost efficient (Cotton *et al.*, 2001). Another benefit of placing UV equipment near the end of the treatment process is that oxidants such as chlorine help increase UV transmittance and make UV more effective (United States Environmental Protection Agency *et al.*, 2003). In general, estimated costs for UV disinfection are lower than estimates for other disinfectants (Cotton *et al.*, 2001; United States Environmental Protection Agency *et al.*, 2003).

1.10 Methods for Viability Determination

Recognising that the terms alive and viable are subject to varying definitions, for the purpose of this thesis, viable is defined as the ability of *Cryptosporidium* to metabolise and transcribe expressed proteins. As mentioned earlier in Section 1.4.2, viability should not always be equated with infectivity (Robertson *et al.*, 1992) and therefore a reliable indicator of oocyst viability is required to then realistically assess potential threats of infection from oocysts.

Research has given different results as to the number of viable oocysts required to infect a healthy person. Gobet and Toze (2001) showed that an infective dose of less than 100 is needed. However, Okhuysen *et al.* (1999) calculated the median infective dose (ID_{50}) to be 132 oocysts (ranging between 9 and 1042 for three different isolates), but with

further data it was recalculated to be 87 oocysts (Okhuysen *et al.*, 1999). The rapid life cycle of *Cryptosporidium* (each parasite can develop and mature within 12-14 hr) and auto-infective cycles contribute to the low dose required for infection (Carey *et al.*, 2004). A sensitive viability determination method is therefore required, since low numbers of oocysts are also generally found in environmental water (Baeumner *et al.*, 2001; Wagner-Wiening and Kimmig, 1995) as well as being required for valid disinfection studies (Fayer *et al.*, 2000). Current techniques for testing oocyst viability are through the use of non-molecular methods such as animal infectivity, tissue culture infectivity, *in vitro* excystation, as well as molecular methods such as fluorogenic dyes, fluorescent *in situ* hybridisation and RT-PCR.

1.10.1 Non-Molecular Methods

1.10.1.1 Animal Infectivity

Neonatal or immunosuppressed mice are used to determine animal infectivity, for example, Widmer *et al.* (1999) has shown that 100 to 10^6 oocysts are required for infection. Mice infectivity models can be used to study the effects of conditions being tested and to monitor oocyst inactivation (Gobet and Toze, 2001; Widmer *et al.*, 1999). Disadvantages associated with mouse infectivity and cell culture when compared with a molecular technique such as PCR or RT-PCR, are in the high expense and labour intensity. These properties are not appropriate for a method used routinely and animal infectivity is not a true measure of human infectivity due to human *Cryptosporidium* isolates not infecting mice effectively (Jenkins *et al.*, 2003; Quintero-Betancourt *et al.*, 2002). Infection of mice by human isolates only occurs when excessively large doses, for example 10^5 oocysts, are administered (Fayer, 1994; Fayer *et al.*, 1996; Harp *et al.*, 1996); this is a larger dose than naturally found in the environment and therefore does not represent a realistic infectious dose. There is also inherent biological variability between litters of neonatal mice, which can make some more or less susceptible to infection (Bukhari *et al.*, 1999).

1.10.1.2 Tissue Culture Infectivity

Cell culture infectivity methods commonly use HCT-8 (Human ileocecal adenocarcinoma), Caco-2 (Human colorectal adenocarcinoma) and MDCK (Madine-Darby Canine Kidney) cell lines to support *Cryptosporidium* and results have shown

equivalence to the 'gold standard' of mice infectivity (Dawson *et al.*, 2004; Rochelle *et al.*, 2002; Slifko *et al.*, 1997). The cell culture method can require PCR to detect viable *Cryptosporidium* at various stages of its life cycle, however no cell culture has been found that can support the development of the entire life cycle. An understanding of oocyst wall-forming protein expression and the assembly into mature walls may help with the development of life cycle completion *in vitro* (Mead *et al.*, 1996). Recently, the use of a fibroblast cell line, MRC-5, has been used successfully to culture *C. parvum* and *C. hominis*; this is important as *C. hominis* oocysts cannot be used in mice infectivity work (Dawson *et al.*, 2004). Dawson *et al.* (2004) used a non-specific auramine phenol staining technique, which fluoresced bright green when life cycle stages were present in the cell monolayer. Cell culture infectivity is expensive, labour intensive and time consuming (peak growth occurs at 48-72 hours) with some isolates not infecting cultured cells (Morgan and Thompson, 1998; Rochelle *et al.*, 1997).

1.10.1.3 In Vitro Excystation

In vitro excystation refers to the rupturing of an oocyst and subsequent release of sporozoites when the oocyst is outside the host organism. It is not an accurate measure of viability or infectious potential as oocysts failing to excyst *in vitro* have been found to be infectious *in vivo* (Fayer *et al.*, 2000). Furthermore, sporozoites can excyst from oocysts through rehydration and appear viable but are not infectious. Another disadvantage of this method is that it requires large concentrations of oocysts (10^5/mL) for analysis and there may be interference from high microbial abundance (Quintero-Betancourt *et al.*, 2002). Another problem encountered with *in vitro* excystation is the lack of specificity for *C. parvum* (Gobet and Toze, 2001).

1.10.2 Molecular Methods

1.10.2.1 Fluorogenic Dyes

Fluorogenic dyes such as propidium iodide (PI: not membrane permeant) and 4,6, diamidino-20-phenylindole (DAPI: membrane permeant), were reported to correlate well with *in vitro* excystation as an indicator of viability (Campbell *et al.*, 1992). However, the two dyes have recently been reported to significantly overestimate oocyst viability (Fayer *et al.*, 2000). Fluorogenic dye assays work on the basis that when sporozoite nuclei take up DAPI but not PI stain they are viable, whereas ones that take

up both dyes are not (Campbell *et al.*, 1992). Jenkins *et al.* (1997) showed that dye permeability is a function of the oocyst wall and not the metabolic activity of the sporozoite; this may explain the overestimation in the number of oocysts considered viable (Jenkins *et al.*, 1997). To demonstrate dye permeability affecting viability results, formaldehyde was used to inactivate oocysts, these did not excyst (indicating non-viability), but when tested using DAPI and PI stains, both formaldehyde-treated and untreated oocysts appeared viable (Jenkins *et al.*, 1997). A disadvantage of fluorogenic dyes is underestimation of the number of oocysts originally present due to the multiple washing and centrifugation steps required for staining, as oocysts can be lost in these processes (Stinear *et al.*, 1996). Also, non-specificity for *C. parvum* and difficulty reading results microscopically due to co-concentrated particles obscuring the view of oocysts (Gobet and Toze, 2001).

1.10.2.2 Fluorescent In Situ Hybridisation (FISH)

Fluorescent *in situ* hybridisation is another method that has been used to determine oocyst viability. Here a fluorescent probe is targeted to 18S ribosomal RNA (rRNA), which makes a good target as it is expected to be present in a high copy number in viable organisms therefore providing a high sensitivity (Fontaine and Guillot, 2003; Quintero-Betancourt *et al.*, 2002; Smith *et al.*, 2004; Vesey *et al.*, 1998). They also have a short post mortem half-life, which proves beneficial for FISH assays as rRNA within non-viable oocysts is degraded due to breakdown in membrane integrity. This prevents dead sporozoites from fluorescing with FISH. However, Smith *et al.* (2004) found that the choice of inactivation was a critical factor in determining whether false-positives were detected or not. For example, Vesey *et al.* (1998) confirmed that under a nonpermeabilising condition such as irradiation with UV light, rRNA could potentially be preserved within the oocyst from RNase damage. Vesey *et al.* (1998) confirmed that without the use of RNase inhibitors when processing environmental samples, membrane damage causes RNA degradation by RNases. FISH may also be affected by high background autofluorescence from minerals or algae in the suspension surrounding the oocysts (Veal *et al.*, 2000).

1.10.2.3 Heat Shock Protein (hsp70) Gene

A variety of PCR tests offer alternatives to conventional diagnosis of *Cryptosporidium* viability (e.g. animal infectivity, *in vitro* excystation and fluorogenic dyes) for both

clinical and environmental specimens. Messenger RNA (mRNA) amplification using RT-PCR is potentially the best method available for viability testing due to its specificity and sensitivity (Gobet and Toze, 2001). Primers are used that are highly specific for *C. parvum* and target the *hsp70* mRNA (Gobet and Toze, 2001). Heat shock proteins are important within biological systems as they help with the correct folding of polypeptides during protein synthesis and are usually present in low amounts (Stinear *et al.*, 1996). To make the method sensitive, oocysts are put under heat shock stress to increase *hsp70* mRNA levels.

The advantage of using the *hsp70* gene is that it is present at all developmental stages of the *C. parvum* lifecycle (de Graaf *et al.*, 1999) and its mRNA is only produced by viable oocysts (Stinear *et al.*, 1996). The key is that although *hsp70* mRNA is highly stable under heat stress, it has a short post mortem half-life, often only a few minutes at ambient temperature (Baeumner *et al.*, 2001), which therefore renders it unlikely to be preserved in non-viable oocysts under environmental conditions (Morgan and Thompson, 1998; Stinear *et al.*, 1996).

Baeumner *et al.* (2001) found the optimal temperature for heat shock stress of *C. parvum* oocysts was 42-43°C for 20 min to gain greatest stimulation of *hsp70* mRNA production. However, Stinear *et al.* (1996) found both the inclusion or exclusion of a heat stress step prior to mRNA extraction had no effect on the expression levels of *hsp70* mRNA in *C. parvum*. This suggested there may be another stress factor present which lead to the expression of *hsp70* mRNA within oocysts. Stinear *et al.* (1996) also noted that exclusion of the heat stress step in favour of a faster overall viability method when using RT-PCR, could result in a compromised detection sensitivity, as by using heat shock an increased chance of detecting small numbers of oocysts is achieved.

Following oocyst lysis, mRNA is specifically isolated (instead of other RNA species), by using magnetic Dynabeads® with short sequences of oligo-dT covalently bound to them that hybridise the polyA tail of mRNA. To test that mRNA and not DNA was specifically captured from *C. parvum* by oligo-$(dT)_{25}$ beads, Stinear *et al.* (1996) subjected the *hsp70* mRNA preparation to both DNase and RNase treatments. The RT-PCR was positive with DNase alone, but negative with RNase treatment, indicating only mRNA was present. Subsequent PCR, using RNase treated RT-PCR product was

negative without further RNase or DNase treatment, also indicating no DNA was present in the original *hsp70* mRNA preparation.

A requirement of working with RNA is a need for strict working conditions therefore all standard operating procedures for RNase reduction should be followed when manipulating RNA. These include using gloves, diethyl pyrocarbonate (DEPC) treatment of water to remove RNases and use of RNase-free aerosol-resistant pipette tips and equipment. Samples should also be processed promptly after oocyst lysis to limit the possibility of mRNA degradation.

Although RT-PCR is rapid, highly sensitive and pathogen specific (Kaucner and Stinear, 1998), it has several limitations. A disadvantage of RT-PCR methods developed to date is that they are a presence/absence test for viability and cannot quantify the number of viable oocysts present. However, Stinear *et al.* (1996) estimated the number of oocysts present by using a visual comparison of agarose gel the band intensity of an internal positive control relative to the *hsp70* RT-PCR product intensity derived from the viable oocysts. On the other hand, Gobet and Toze (2001) has shown that mRNA detection can be difficult to interpret and amounts detected can be dependent on which mRNA extraction method is used (Gobet and Toze, 2001). It has also been noted that quantitation of eukaryotic mRNA such as *hsp70* transcripts would be difficult as they undergo a 1000 to 10 000-fold increase under heat shock stress conditions (Chung *et al.*, 1999; Stinear *et al.*, 1996).

False positives can result from detection of naked nucleic acids in a sample suspension and laboratory contamination. False negatives can arise when an adequate positive control is not used throughout the RT-PCR method. Negative results can also be due to the co-concentration of PCR inhibitors from the environment (e.g. humic acids, salts and heavy metals) during oocyst isolation and purification (Stinear *et al.*, 1996). It has been suggested that when using RT-PCR on faecal samples, which often contain large numbers of oocysts, dilution or incorporation of a DNase treatment may be necessary for mRNA isolation. This precaution prevents a carryover of oocyst DNA and increases the quantity and quality of mRNA isolated as a result of less competition during isolation (Stinear *et al.*, 1996). Therefore, for routine acceptance of any PCR-based technique as a diagnostic tool, inhibition must be overcome and a standardised, reliable

method of recovering oocysts from water supplies must be developed. Although false results are never desirable with any testing system, when public health is a concern, overestimation of viability may be preferable to underestimation or false-negative results (Baeumner *et al.*, 2001; Gobet and Toze, 2001). However, false positives can cause economic loses and public panic.

1.10.2.4 Other Molecular Methods

The *hsp70* gene has many qualities which make it a good target to use for determination of oocyst viability using RT-PCR. However, there are other gene targets which are also worthy of consideration. Jenkins *et al.* (2000) used RT-PCR directed at amyloglucosidase, the enzyme that breaks down amylopectin, as a viability marker. The rationale for this research was based on the finding of Fayer *et al.* (1998) that non-infectious *C. parvum* oocysts contain negligible amounts of amylopectin (a known energy store for sporozoites within oocysts). Jenkins *et al.* (2000) found amyloglucosidase mRNA levels correlated with parasite infectivity in neonatal mice. Widmer *et al.* (1999) investigated whether the ß-tubulin gene could be used as a marker for viability also. This gene was chosen, as at this time it was believed to be the only primary transcript in the *C. parvum* genome containing an intron. Abrahamsen *et al.* (2004) subsequently sequenced the *C. parvum* genome and predicted a significant increase in the number of genes containing introns, 5% of the total 3807 genes present. An intron is a useful feature of a gene for experimental reasons. It allows the elimination of interference from DNA in the PCR after the reverse transcription when primers are designed to amplify either side of the intron, resulting in a product that is assured of originating from mRNA (Widmer *et al.*, 1999).

Fontaine and Guillot (2003) used real-time RT-PCR to investigate the use of 18S rRNA as a viability marker. They found that 18S rDNA was more heat-stable than 18S rRNA after oocyst lysis and they hypothesised that the oocyst wall and ribosomal subunits remain intact at 95°C and therefore that 18S rRNA may not be associated with viability after heat treatment (Fontaine and Guillot, 2003).

Wagner-Wiening and Kimmig (1995) used PCR to detect *C. parvum* and by incorporating *in vitro* excystation prior to the PCR they determined oocyst viability. This was based on the foundation that DNA can be detected after the release of

sporozoites from oocysts (excystation) and that active excystation demonstrates viability (Wagner-Wiening and Kimmig, 1995). However, controls were not carried out to prove excystation does in fact imply viability.

1.11 Oocyst Recovery Using Immunomagnetic Separation (IMS)

In order to remove unwanted humic acids and other enzyme inhibitors that are problematical for PCR reactions, IMS is used in the *hsp70* RT-PCR technique. IMS serves many functions including isolation, concentration and purification of specific organisms or nucleic acid sequences. For example, to capture *Cryptosporidium* oocysts, paramagnetic polystyrene beads coated with monoclonal antibody to *Cryptosporidium* oocyst wall antigens are used; these only attach to intact oocysts and help to isolate them from environmental water samples and faecal matter. The oocysts are isolated from the sample by using a magnet and the unwanted surrounding material is removed by washing. This serves to concentrate oocysts from environmental samples and leaves small volumes ideal for PCR methods (Rochelle *et al.*, 1999). Recovery efficiencies from water concentrates are dependent on the mechanism of agitation during the magnetic capture procedure however average yields are between 60 and 95% (Dynal Biotech, 2002). Oocyst isolation methods, which do not use the IMS system, have low and variable recovery rates ranging from 0 to 140%, these losses often occur during filtration and centrifugation steps (Rochelle *et al.*, 1999). Therefore, it is necessary to use a system that reduces these losses. It has been noted that dead oocysts might be preferentially concentrated when using density gradient centrifugation during isolation from a sample, so this must be taken into account when designing a method (Jenkins *et al.*, 2003; Stinear *et al.*, 1996). Percoll, salt and sucrose gradients are also used to separate *Cryptosporidium* oocysts, however it is not known to what extent these compounds have on the viability of oocysts (Ionas, 2002).

Thus, IMS is a powerful tool for water microbiology as it is simple to use, does not require expensive equipment and effectively separates the specified organism from the sample matrix (Kaucner and Stinear, 1998). In addition, specific nucleic acid sequences have been targeted recently using magnetic beads that have oligonucleotides attached (Stinear *et al.*, 1996).

1.12 The Effect of Temperature and Desiccation on Oocyst Viability

Research has been conducted to test various temperatures and environmental conditions under which oocysts can survive (Fayer *et al.*, 2000; Harp *et al.*, 1996; Kim and Healey, 2001; Robertson and Gjerde, 2004). However, viability determination in these experiments has been through the use of animal infectivity and not RT-PCR, therefore necessitating research that uses RT-PCR to confirm, compare or disprove these findings.

Oocysts of *C. parvum* can remain viable for many months and even years depending on the environmental conditions that they are subjected to. It has been found that storage temperatures below 0°C results in oocysts surviving for shorter periods of time than they would at higher temperatures (Fayer and Nerad, 1996; Fayer *et al.*, 1996). This is due to ice crystal formation where membrane disruption occurs, rupturing oocysts, which render them non-viable. Viability of oocysts also depends on the medium in which the oocysts are stored in, for example water, faecal matter or cryopreservatives. The addition of dimethyl sulphoxide (DMSO) to the oocyst preparation helps to reduce ice crystals forming as it is a cryoproservative. Despite *hsp70* mRNA being highly stable during heat stress, determining its survival within oocysts under other temperature conditions is necessary, to ensure that the viability-indicating RT-PCR products correlate with the presence of viable oocysts (Stinear *et al.*, 1996).

Fayer *et al.* (1998) found a large proportion of oocysts, held at 20°C for 6 months, were still infectious for suckling mice. Whereas other oocysts held at 25°C and 30°C were only infectious up to 3 months, as these higher temperatures resulted in more rapid loss of viability. Even a 20 minute period in which oocysts were warmed from 9°C to 55°C resulted in loss of infectivity for suckling mice (Fayer *et al.*, 2000). Oocysts held at 59.7°C for 5 min had very low infectivity and others held at 71.7°C for 15 sec were killed (Harp *et al.*, 1996). These temperature/time combinations were specifically tested as they are used in high-temperature-short-time (HTST) commercial pasteurisation of milk and water (Harp *et al.*, 1996).

Snap freezing and programmed freezing to -70°C resulted in immediate killing of *C. parvum* oocysts even in the presence of a variety of cryoproservatives, including DMSO and glycerol (Fayer *et al.*, 1996). These findings suggest that fluids within oocysts offer minimal cryoprotection to the sporozoites. However, Kim and Healey (2001) found that when oocysts were cryopreserved in faeces, between 11.7% and 34% of them were viable and infective to mice after 30 days at -20°C. This suggests that faecal matter may act as an effective cryoprotectant. However, this determination of viability was done using DAPI staining and it was postulated by Robertson *et al.* (1992) that faecal matter may reduce the permeability of the oocyst to DAPI and therefore an underestimation of the number of viable oocysts present could occur.

Under freezing conditions, survival of oocysts is higher when the temperature increase is achieved slowly and does not go to extreme freezing levels. For example, some oocysts stored in water at -20°C were viable for up to 8 h, but not at 24 h (Fayer *et al.*, 2000). This result suggests a lower level of viability when oocysts are stored in water compared to when stored in faeces as found by Kim and Healey (2001). In another experiment, oocysts held at -10°C were infectious to mice up to 1 week after storage, whereas those held at -5°C remained viable for up to 2 months (Fayer *et al.*, 2000). It would be of interest to apply the RT-PCR method, since it is a more sensitive test, to determine viability in this situation and observe whether there was an increase in the estimated number of resistant oocysts. Robertson *et al.* (2004) also showed that fluctuating environmental temperatures (ranging from -9°C and 9°C), causing freeze/thaw cycles, had a deleterious effect on *Cryptosporidium* oocysts through the increasing permeability to propidium iodide dye. Desiccation was investigated by Robertson *et al.* (1992) and was found to be lethal to oocysts; only 3% of oocysts were found to be viable after 2 h of desiccation through air drying at room temperature and 100% killing was reported at 4 h.

1.13 Objectives of Thesis

Research conducted in this study aims to achieve the optimisation of an RT-PCR assay using *hsp70* mRNA expression for the determination of *C. parvum* oocyst viability. The assay will be based on an RT-PCR method to avoid the limitations of other viability methods which are not as sensitive, lack specificity for *C. parvum* and/or are too expensive and labourious. *Cryptosporidium* is highly chlorine resistant and difficult to filter out of water because of its small size, therefore UV light has been tested as an alternative for oocyst inactivation. However, the effectiveness of UV light to inactivate *Cryptosporidium* has previously been determined by viability methods other than RT-PCR. This necessitates testing viability using an RT-PCR oocyst viability assay to investigate whether these previous findings can be corroborated. A cidal UV dose will be determined and if the sensitivity of oocyst detection for the RT-PCR oocyst viability assay is low, a 3-log reduction will be sought. Once a cidal UV dose for *C. parvum* is found, the phenomenon of photoreactivation will be also investigated.