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

A thesis presented in partial fulfilment of the
requirements for the degree of
Master of Science
in
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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 immunocompetent people, but can be life-threatening to immunocompromised 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/cm²; 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/cm² 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
<i>hsp70</i>	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 (Baumner *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).

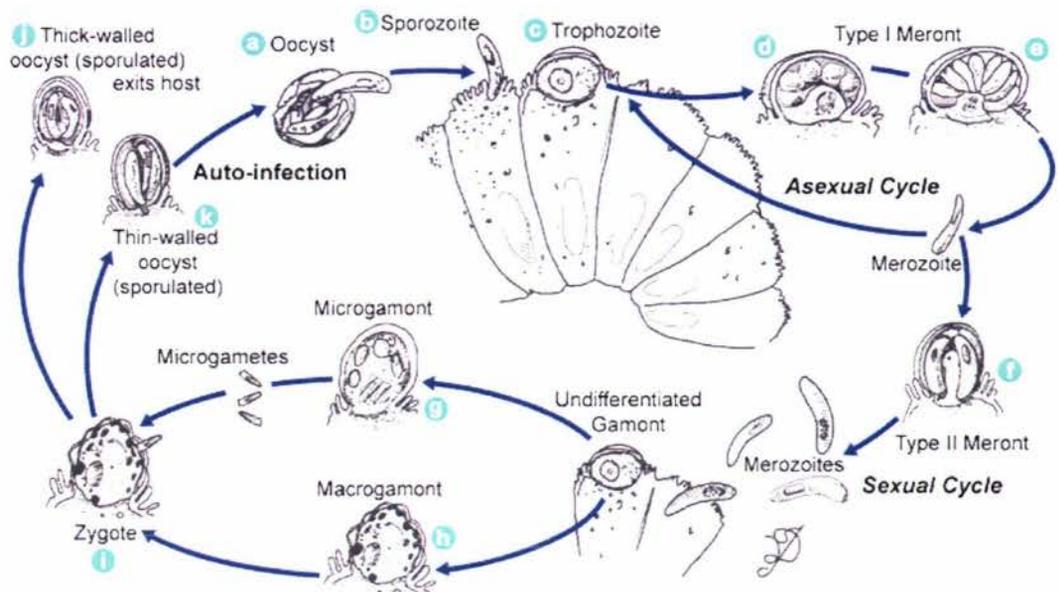


Figure 1.1: *Cryptosporidium* lifecycle. Key: (a) excystation of the oocyst, (b, c) sporozoites released and parasitise epithelial cells of gastrointestinal tract, (d, e, f) parasites undergo asexual multiplication (schizogony or merogony), followed by sexual multiplication (gametogony) where (g) microgametes (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* (Nwachuku 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/cm² whereas Campbell *et al.* (1995) used fluorogenic dyes

and excystation achieving only a 2- to 3-log reduction with 10 000 mJ/cm². Surprisingly, this is approximately a 10⁴-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/cm² 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/cm²) 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/cm² 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/cm² for fresh and 14 mJ/cm² 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 Biosimulators 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 (biosimulators) 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/cm² (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 (Nwachuku 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 (Nwachuku 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 <i>et al.</i> , (1995)	Fluorogenic dyes & excystation	10 000	2-3
Bukhari <i>et al.</i> , (1999)	Mice infectivity	41	4
Clancy <i>et al.</i> , (2000)	Mice infectivity	6	3
Drescher <i>et al.</i> , (2001)	Mice infectivity	120	5.4
Shin <i>et al.</i> , (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 ($1 \text{ mJ}/\text{cm}^2 = 1 \text{ mWs}/\text{cm}^2$) (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).

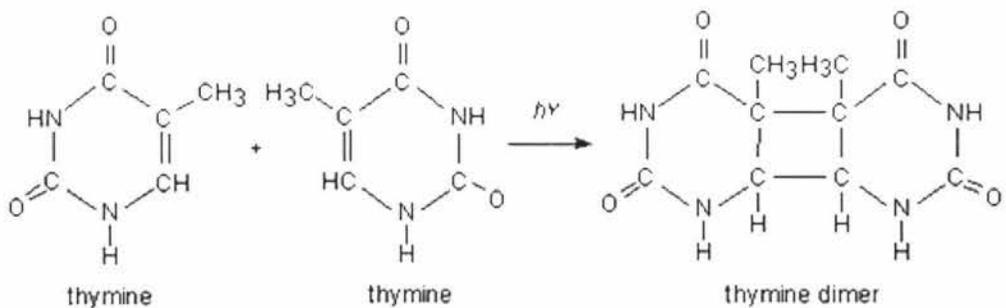


Figure 1.2: Photochemical dimerisation of two thymine bases to form a thymine dimer (taken from Bolton (2000) (Bolton, 2000))

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 4×10^5 -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/cm² (required to inactivate viruses) and a dose of 40 mJ/cm² (chosen as a conservative requirement to

inactivate *Cryptosporidium*) and showed a 50% increase in capital costs with 140 mJ/cm² (Cotton *et al.*, 2001). It has been estimated that at 40 mJ/cm², 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/cm² 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 (ID₅₀) 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 losses 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 β -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 cryopreservative. 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 cryoprotectants, 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 tidal 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 tidal UV dose for *C. parvum* is found, the phenomenon of photoreactivation will be also investigated.

CHAPTER 2 : MATERIALS AND METHODS

The methods outlined in Sections 2.1 to Section 2.7 describe general procedures and components used in the development of the RT-PCR oocyst viability assay. Applications of the assay are described in Sections 2.8 and 2.9.

2.1 *Cryptosporidium* Strains and Purification of Oocysts

2.1.1 Strains of Oocysts

Viable *Cryptosporidium parvum* oocysts of Camden (BTF Pty Ltd, North Ryde, NSW, Australia) and Iowa (Waterborne™ Inc, New Orleans, USA) strains were used in this study; both strains were of the bovine genotype. These strains were imported as a viable and homogenous supply of oocysts as required because extraction from New Zealand human or cattle faeces could not satisfy these criteria.

2.1.2 Purification of Oocysts

2.1.2.1 Imported Oocysts

C. parvum (Camden strain) oocysts, deemed to be 70% viable for 3 months when stored at 4°C, were purified by BTF Pty Ltd using density gradient centrifugation. *C. parvum* (Iowa strain) oocysts were purified by Waterborne™ Inc using sucrose and Percoll gradient centrifugation and water washes. Oocysts were supplied in phosphate buffered saline containing penicillin, streptomycin, gentamicin, and amphotericin B antibiotics.

2.1.2.2 Oocyst Extraction from Faeces

Approximately 5 g of human (sourced from NZ Medical Laboratories) or calf (from Dairy Number 1, Massey University, Palmerston North, NZ) faeces were emulsified in a 15 mL tube containing 3 mL of 1× PBS (Appendix 1.5). The total volume was made up to 7 mL using 1× PBS, shaken vigorously and vortexed. The suspension was strained into a fresh

15 mL tube with the original tube being rinsed with 2 mL of distilled water. Three millilitres of di-ethyl ether (Sigma-Aldrich Pty. Ltd, Sydney, Australia) was added to the suspension, shaken vigorously for 1 minute and centrifuged for 1 minute at 2100 rpm. The supernatant and subsequent lipid layer was removed, leaving approximately 500 µl, which was used to resuspend the pellet containing the oocysts.

2.2 Procedures Using Oocysts

2.2.1 Enumeration of Oocysts

Serial dilutions of oocysts in DEPC-treated water (Section 2.4.2) were performed as required, to obtain a range of *C. parvum* oocysts between 5×10^4 and 1×10^1 for use in oocyst detection sensitivity, cidal UV dose and photoreactivation trials (Sections 2.8 and 2.9). Two methods were used to enumerate the oocysts; haemocytometer chamber counts and monoclonal antibodies labelled with fluorescein isothiocyanate (FITC).

2.2.1.1 Haemocytometer Chamber

Haemocytometer chamber counts were performed in accordance with USEPA Method 1622, version 2001 (United States Environmental Protection Agency, 2001a). Ten microlitres of diluted *Cryptosporidium* oocysts was loaded onto a clean haemocytometer and using a light microscope at 200x magnification, the four corners of the chamber (each 1mm^2) were counted. Six separate chamber counts were averaged and the number of oocysts per mL was calculated using a formula derived by the USEPA (Appendix 4.1).

2.2.1.2 Fluorescent Labelling

The higher dilutions of oocysts, used for oocyst detection sensitivity trials, were counted using a FITC-labelled monoclonal antibody, as this method was limited to small numbers of oocysts. A 50 µl aliquot of diluted *Cryptosporidium* oocysts was placed on a three-well SuperStick™ Slide (Waterborne™ Inc) and left at room temperature until dry (approximately 45 minutes). Fifty microlitres of absolute methanol (Sigma-Aldrich Pty. Ltd) was placed

onto the slide to fix the specimen, as well as to assist with the permeability of the oocyst, and left for approximately 20 minutes at room temperature until dry. A 50 µl aliquot of FITC-labelled *Cryptosporidium*-specific IgG1 monoclonal antibodies (EasyStain™ C&G FITC, BTF Pty Ltd, NSW, Australia), diluted 2-fold, was spread over the sample and incubated at room temperature in a dark humidity chamber for 30 minutes. Excess antibody was removed from the slide with wash buffer and a drop of mounting medium (to reduce fading of the FITC stain) was placed on the slide, covered with a coverslip and sealed with clear nail polish. The sample was examined using an Olympus BX60 epifluorescent microscope (Olympus Corporation) at 200x magnification and *Cryptosporidium* oocysts (approximately 4-6µm in size) were detected by the characteristic apple-green fluorescence.

2.2.2 Immunomagnetic Separation (IMS) of Oocysts

Cryptosporidium oocysts were concentrated using the Dynabeads® anti-*Cryptosporidium* kit (Dyna® Biotech, Oslo, Norway). This product has been evaluated by the USEPA and was determined to be an integral part of Method 1623 (United States Environmental Protection Agency, 2001b). Dynabeads® are microscopic, paramagnetic beads with species-specific antibodies covalently bound to their surface.

IMS was carried out in a PCR tube using 100 µl of Dynabeads® anti-*Cryptosporidium* (Dyna® Biotech) following the manufacturers protocol. The tube was rotated at 8 rpm for one hour using the LABQUAKE® rotisserie (Barnstead/Thermolyne, Iowa, USA) to ensure a Dynabead®/oocyst complex formed. To prevent oocyst losses, the oocysts were not detached from the Dynabeads®.

2.2.3 Heat Shock of Oocysts

Oocysts were heat shocked at 45°C for 12 minutes to facilitate the over-expression of *hsp70* mRNA, which allowed its detection to be more sensitive. The heat shocked oocysts were stored at 4°C until required.

2.2.4 Lysis of Oocysts

2.2.4.1 Freeze/Thaw Method

Oocysts were collected by centrifugation at 10 000x g for 15 minutes, the supernatant discarded and the oocysts resuspended in 100 µl of 1% Nonidet P40/TE Buffer (Roche Diagnostics) (Appendix 1.4) and 20 µl of 20% Chelex[®] (BioRad) (Appendix 1.6). The oocysts were cycled four times between liquid nitrogen for 1 minute and boiling water for 1 minute.

2.2.4.2 Cell Lysis Buffer Method

The *Cryptosporidium* oocyst/IMS bead complex was captured against the wall of a microcentrifuge tube using a magnetic tube holder (Appendix 3.1) and the supernatant was aspirated. Twenty microlitres of ice-cold Cell Lysis II Buffer (Cells-to-cDNA™ II kit, Ambion Inc.) was added to the sample of oocysts, which was then immediately placed in a thermal cycler at 75°C for 20 minutes to facilitate oocyst lysis and inactivate RNases.

2.3 Isolation of Nucleic Acid and mRNA

2.3.1 Nucleic Acid Isolation

Following lysis by freeze/thaw (Section 2.2.4.1), the oocysts were centrifuged at 10 000x g for 2 minutes at room temperature and the supernatant containing the nucleic acid was transferred to a new 600 µl tube (with care not to transfer any of the pellet, as the Chelex[®]

inhibits PCR amplification). The extracted nucleic acid was stored at 4°C until required (Section 2.6.2, 2.6.3 or 2.6.4.2).

2.3.2 mRNA Isolation

Dynabeads[®] Oligo (dT)₂₅ (DynaL Biotech) facilitate the isolation of mRNA from *C. parvum* oocysts due to the covalently bead-bound oligo-dT hybridising to the polyA tail of mRNA. *C. parvum* mRNA was isolated using the Dynabeads[®] mRNA DIRECT™ Kit (DynaL Biotech). Fifty microlitres of resuspended Dynabeads[®] Oligo (dT)₂₅ was transferred to an RNase-free microcentrifuge tube, placed in a magnetic tube holder (Appendix 3.1) and the supernatant removed. The beads were prewashed in 280 µl Lysis/Binding buffer containing an RNase inhibiting agent to allow for isolation of intact mRNA.

Oocyst lysate was added to the Dynabeads[®] and the tube was rotated on the LABQUAKE[®] rotisserie (Barnstead/Thermolyne) at room temperature for 20 minutes to allow the Dynabeads[®] to anneal to the mRNA. The sample tube was placed in a magnetic tube holder (Appendix 3.1), gently rocked from end to end for 2 minutes to capture the Dynabead[®]/mRNA complex and the supernatant removed. The Dynabead[®]/mRNA complex was washed twice with 500 µl Washing Buffer A and once with 300 µl Washing Buffer B. The magnetic tube holder (Appendix 3.1) was used in the washing process to capture the Dynabead[®]/mRNA complex when removing the supernatant to ensure the supernatant was fully removed after each washing step to prevent carryover of contaminating DNA.

To elute the mRNA from the Dynabeads[®], 100 nmol Elution Buffer was added to the Dynabead[®]/mRNA complex and incubated at 70°C for 2 minutes in a thermal cycler (GeneAmp[®] PCR System 9700, version 3.01 software, PerkinElmer Inc.). The supernatant containing the mRNA was transferred to a new PCR tube using a magnetic tube holder (Appendix 3.1) to separate the Dynabeads[®] Oligo (dT)₂₅ from the supernatant.

2.4 Precautions for RNA Work

2.4.1 Equipment Precautions

RT-PCR experiments were performed in a safety cabinet that was wiped down with RNase-Off (CPG Inc[®], NJ, USA). Gloves were worn at all times and were changed regularly. RNA-only pipettes and barrier tips were used exclusively to protect RNA from degradation.

2.4.2 Diethyl pyrocarbonate (DEPC)-Treated Water

Diethyl pyrocarbonate (DEPC) was used to deactivate any RNases present in the water. 0.01% DEPC (v/v) was added to water and incubated at 37°C overnight. The solution was autoclaved twice to remove the DEPC to prevent further downstream PCR inhibition.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Oligonucleotides

The custom oligonucleotides (Invitrogen Corporation) used in this study (Table 2.1) were made to 1 nmol/μl in sterile TE buffer (Appendix 1.3) for a stock solution and the working stock had a final concentration of 20 pmol/μl. All primer solutions were stored at -20°C until required.

2.5.2 General PCR Conditions

PCR reactions were performed using DNA from approximately 1.25×10^4 *C. parvum* oocysts with *Taq* DNA Polymerase (Qiagen) and the included kit buffers according to manufacturer's recommendations. Cycling conditions consisted of an initial denaturation step of 94-98°C for 2-5 minutes followed by 30-45 cycles of: 94-98°C for 30 seconds, 55-

61°C for 30 seconds and 65-72°C for 30-90 seconds (extension temperature dependent on polymerase used), followed by a final extension step (at the same temperature used in the previous extension step) of 5 minutes. The specific annealing temperature used for each primer pair is indicated in Table 2.1. The thermal cycler used was the GeneAmp® PCR System 9700 with version 3.01 software from PerkinElmer Inc.

Table 2.1: Oligonucleotides used in this study

Primer	Sequence	Annealing Temperature (°C)	PCR Product Size (bp)	Gene	Reference
Forward (Cphsp 2423)	AAATGGTGAGCAATCCTCTG	55	361	<i>hsp70</i>	[Rochelle, 1996 #334]
Reverse (Cphsp 2764)	CTTGCTGCTCTTACCAGT AC				
Forward (HSP FO)	CAAGGATGCTGGTATGGACA	57.5	1020	<i>hsp70</i>	[Chambers, 2002 #566]
Reverse (HSP RO)	AGATCCTGGCATAACCACCTG				
Forward (HSP FI)	TGATGGTATTCCACCAGCAC	59	213	<i>hsp70</i>	[Chambers, 2002 #566]
Reverse (HSP RI)	CTGCTCATCCTCACCCCTTGT				
Forward (CHSP 1)	AGCAATCCTCTGCCGTACAGG	61	590	<i>hsp70</i>	[Kaucner, 1998 #435]
Reverse (CHSP 4)	AAGAGCATCCTTGATCTT CT				
Forward (TUB 1)	ATGAGAGAAATTGTTTCATGTT	55	592	β -tubulin	[Caccio, 1999 #56]
Reverse (TUB 2)	AAAGGTCTGCAAATAACGAT C				
Forward (GHSP Forward)	AGGGCTCCGGCATAACTTTCC	55	163	<i>hsp70</i>	[Abbaszadegan, 1997 #565]
Reverse (GHSP Reverse)	GTATCTGTGACCCGTCCGAG				

Primer sequences are shown in the 5' to 3' direction

2.5.3 Reverse Transcription PCR (RT-PCR)

2.5.3.1 One-Step RT-PCR

One-step RT-PCR was performed as follows; each sample reaction contained 1x HotMaster™ *Taq* Buffer (Eppendorf), 10 pmol of each dNTP, 10 U RNase Inhibitor (Cells-to-cDNA™ II kit, Ambion Inc.), 20 pmol of each primer (Table 2.1), 100 U M-MLV Reverse Transcriptase (Cells-to-cDNA™ II kit, Ambion Inc.), 4 U HotMaster™ *Taq* DNA Polymerase (Eppendorf), 10 µl of either sample mRNA or nuclease-free water (for the minus-template negative control) and nuclease-free water to 25 µl total volume. For the Armoured® RNA positive control, 3 µl of Armoured RNA® control (Cells-to-cDNA™ II kit, Ambion Inc.) was added to 20 µl Cell Lysis II Buffer (Ambion Inc.) and was heated at 75°C for 20 minutes. Ten picomoles of Armoured® RNA Primer Pair (Cells-to-cDNA™ II kit, Ambion Inc.) was used. An RT-minus negative control, a reaction excluding reverse transcriptase but containing *Taq* DNA Polymerase, which confirms the product arose from template and not genomic DNA, was also included for each experiment.

Cycling conditions were: 42°C for 15 minutes, 94°C for 2 minutes and then 44 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 65°C for 90 seconds followed by a final extension at 65°C for 5 minutes and held at 4°C until required.

2.5.3.2 Two-Step RT-PCR

The RETROscript® First Strand Synthesis Kit (Ambion Inc.) was used for cDNA synthesis. Each 16 µl sample reaction contained 100 pmol Oligo (dT)₁₈ primer (RETROscript® First Strand Synthesis Kit, Ambion Inc.), 10 pmol dNTPs (RETROscript® First Strand Synthesis Kit, Ambion Inc.) and 10µl of sample mRNA. The positive control contained the same components as the samples but instead used 1 µg Control Template RNA (RETROscript® First Strand Synthesis Kit, Ambion Inc.). These were incubated at 70°C for 3 minutes in a thermal cycler (GeneAmp® PCR System 9700, version 3.01 software, PerkinElmer Inc.).

The following were then added to each sample including the positive and negative controls: 1x RT buffer, 10 U RNase Inhibitor, 100 U MMLV-Reverse Transcriptase (RETROscript® First Strand Synthesis Kit, Ambion Inc.) and nuclease-free water to a 20 µl total volume. Cycling conditions were: 43°C for 60 minutes followed by 92°C for 10 minutes, with a final holding temperature of 4°C.

PCR amplification of the cDNA was performed using the following reagents and conditions: 1x buffer (*Taq* DNA Polymerase Kit, Qiagen, Biolab Ltd, Auckland, NZ), 1 µl dNTPs (Roche Diagnostics), 2 µl MgCl₂ (*Taq* DNA Polymerase Kit, Qiagen, Biolab Ltd, Auckland, NZ), 8 µl Q buffer (*Taq* DNA Polymerase Kit, Qiagen, Biolab Ltd, Auckland, NZ) and 1 U *Taq* DNA Polymerase (Qiagen, Biolab Ltd, Auckland, NZ). Primers (Table 2.1) were added: 20 pmol CHSP1 primer and 20 pmol CHSP4 primer to all sample tubes, RT minus control and negative control; 20 pmol GHSP Forward primer and 20 pmol GHSP Reverse primer to the DNA positive control and 12.5 pmol Control PCR primers (RETROscript® First Strand Synthesis Kit, Ambion Inc.) to the positive control. Template was added as required and was either 2 µl cDNA from the above RT-PCR or 2 µl of 1:100 *Giardia intestinalis* Trophozoite genomic DNA for the DNA positive control or mRNA from a recent oocyst extraction stored at -80°C for the RT minus control.

Cycling conditions were: 98°C for 5 minutes and then 45 cycles of 98°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes and finally held at 4°C until required.

2.6 Procedures Using Nucleic Acid

2.6.1 DNaseI Treatment of mRNA

Contaminating DNA present after mRNA extraction was removed by DNaseI treatment. Two U DNaseI (DNA-free™, Ambion Inc.) and 1x DNase Buffer (DNA-free™, Ambion Inc.) was added to 10 µl of extracted mRNA eluted from the Dynabeads® Oligo (dT)₂₅

(Section 2.3.2) in a total reaction volume of 12 μ l. The sample was incubated at 37°C for 20 minutes then 5 μ l of DNase Inactivation Reagent (DNA-free™, Ambion Inc.) was added, incubated at room temperature for 2 minutes and centrifuged at 10 000x g for 1 minute. The supernatant containing mRNA was collected and used in reverse transcription polymerase chain reactions (RT-PCR) (Section 2.5.3.1).

2.6.2 Agarose Gel Electrophoresis

PCR and RT-PCR amplified products were detected by electrophoresis through 1.5-2% agarose (Roche Diagnostics) gels in 1x sodium boric acid buffer (Appendix 1.2) or 1x TAE buffer (Appendix 1.1). Five microlitres of PCR product containing 1x gel loading dye (Appendix 1.7) were loaded into individual lanes and electrophoresed using a horizontal gel electrophoresis system (Horizon™ 58, Gibco BRL, Invitrogen Corporation) for 20 minutes at 300-350 V for sodium boric acid buffer gels or 2 hours at 80 V for TAE buffer gels. The 1 Kb Plus DNA Ladder™ (Invitrogen Corporation) was used as a molecular size marker. The gel was stained in ethidium bromide (5 μ g/mL) for 10 minutes, destained in 1x TAE Buffer (Appendix 1.1) for 5 minutes and the DNA visualised under ultraviolet light.

2.6.3 Genotyping of *C. parvum* (Camden Strain) by Restriction Fragment Length Polymorphism (RFLP) - PCR Analysis

RFLP-PCR analysis was used to determine the genotype of *C. parvum* (Camden strain) oocysts. PCR (Section 2.5.2) was carried out using TUB 1 & 2 primer pair specific for the *C. parvum* β -tubulin gene (Table 2.1). The resulting 592 bp product was digested separately using 10U *DdeI* (Roche Diagnostics) and 5U *HaeIII* (Biolab Ltd, Auckland, NZ) restriction endonuclease enzymes according to the manufacturers instructions. The bovine genotype resulted in two products with each enzyme digest, 414 bp and 178 bp with *DdeI* and 380 bp and 212 bp with *HaeIII*, whereas the human genotype would not digest with *DdeI* and would result in two products, 362 bp and 228 bp, with *HaeIII*.

2.6.4 DNA Purification

2.6.4.1 Agarose Gel Purification of DNA Fragments

DNA fragments were purified from agarose gel slices using QIAquick Gel Extraction Kit (Qiagen, Biolab Ltd, Auckland, NZ) as per the manufacturers protocol.

2.6.4.2 Purification of DNA from a PCR Reaction

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) as per the manufacturers protocol.

2.7 DNA Sequencing and Computer Analysis

2.7.1 Sequencing of the *hsp70* PCR Product

The *hsp70* PCR products made using the Cphsp 2423 & 2764 primer pair (Table 2.1) from both the Camden and Iowa strains were purified prior to sequencing from either an agarose gel (Section 2.6.4.1) or from a PCR reaction (Section 2.6.4.2). The DNA concentration was determined by comparing the band intensity of the PCR product in an agarose gel (Section 2.6.2) to the band intensities in the Low DNA Mass™ Ladder (Invitrogen Corporation). The quantity of purified DNA required for sequencing was dependent on the size of the PCR product (1ng/100bp) and the concentration of DNA. An aliquot containing 3.2 pmol of each primer (Cphsp 2423 & 2764, Table 2.1) was used to sequence the PCR product, processed at the Allan Wilson Centre Genome Service, Massey University, Palmerston North, NZ, using a capillary ABI3730 Genetic Analyser (Applied Biosystems Inc, Perkin Elmer, CA, USA).

2.7.2 Sequence Analysis

Sequences were manually inspected and aligned using MT Navigator, Version 1.0.2b.3 (Applied Biosystems Division, Perkin Elmer). The Basic Local Alignment Search Tool

(BLAST) (US National Library of Medicine, USA. www.ncbi.nlm.nih.gov/BLAST/) was used to confirm the PCR amplicon was specific for *C. parvum* Heat Shock Protein 70 gene.

2.8 Determination of the Cidal Ultraviolet (UV) Dose for *Cryptosporidium* Oocysts

Determination of the cidal UV dose for *C. parvum* oocysts was conducted using a collimated beam apparatus housing a UV lamp, which emitted light at the germicidal wavelength of 254 nm (Figure 2.1). Irradiated oocysts were tested for viability using the optimised RT-PCR oocyst viability assay as outlined in Sections 2.2.4.2, 2.3.2, 2.6.1, 2.5.3.1 and 2.6.2.

2.8.1 Apparatus

A collimated beam apparatus was constructed by the Institute of Fundamental Sciences Engineering Services Workshop, Massey University, Palmerston North and consisted of a metal fabrication with two polyvinyl chloride collimating tubes attached, each of which had an inner diameter of 6.5 cm and a length of 40.6 cm. The 254 nm UV lamp (Spectronics Corporation, NY, USA) (Appendix 3.2) sat at the top of the apparatus with a metal shutter below it to control exposure times.

2.8.2 Procedure

The UV lamp was switched on, with the shutter closed, for 30 minutes prior to irradiation to stabilise the lamp output. Irradiance from the UV lamp was determined by placing the 254 nm UV detector (International Light, Newburyport, USA) (Appendix 3.4), attached to the radiometer (Model 1400A, International Light, Newburyport, USA), underneath each of the collimated tubes and readings were measured according to the manufacturers protocol. A reading was taken before the sample was irradiated and used to calculate the length of time required for the dose being tested (Appendix 4.2). After oocyst irradiation, UV

irradiance readings were also taken to check for consistency of the lamp output. An overall mean irradiance of the oocysts was calculated by averaging the before and after readings.

Duplicate glass petri dishes (59 mm diameter) for each UV dose tested (40 mJ/cm² to 1100 mJ/cm²) were filled with 10 mL of DEPC-treated water (Section 2.4.2) (which had previously had a turbidity reading taken, Appendix 2), a magnetic stir bar (2mm x 7mm, HACH Company, USA) and 5x10⁴ viable oocysts (Section 2.2.1.1). Each petri dish was placed on a magnetic stirrer, under a collimated tube and the suspension was mixed which ensured even UV irradiation of the oocysts. The collimated tubes were lowered to cover each dish during the UV exposure when the shutter was open.

IMS (Section 2.2.2) was used to recover the oocysts from the petri dish and reduce the sample volume from 10 mL down to 100 µl. Specifically, the UV irradiated oocyst suspension was pipetted into a disposable 15 mL plastic tube and 100 µl Dynabeads[®] anti-*Cryptosporidium* was added. The sample tube was rotated for 1 hour (Section 2.2.2), placed in a magnetic tube holder (Appendix 3.1), gently rocked from end to end for 1 minute and the supernatant was removed. One mL DEPC-treated water was used to resuspend the oocyst/bead complex that was then transferred to a 1.5 mL microcentrifuge tube, the Dynabeads[®] were magnetically captured and the supernatant removed. One hundred microlitres of DEPC-treated water was used to resuspend the oocyst/bead complex and this was transferred to a 0.2 mL PCR tube. The sample was heat shocked (Section 2.2.3) and processed through the RT-PCR oocyst viability assay as outlined in Sections 2.2.4.2, 2.3.2, 2.6.1, 2.5.3.1 and 2.6.2. A sample was deemed viable when the 361 bp PCR product (using the Cphsp 2423 & 2764 primer pair) was present and all controls worked correctly (Figure 3.2).

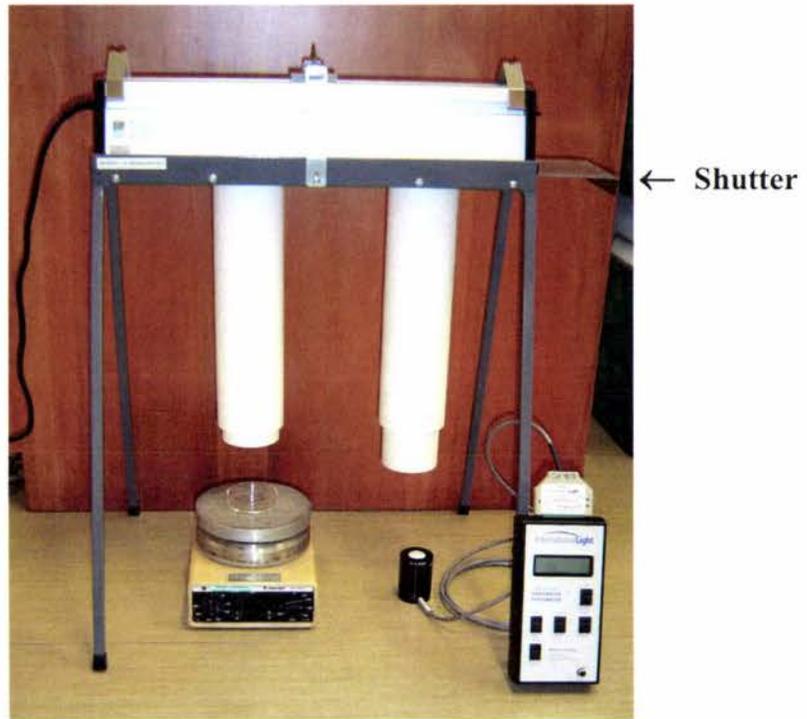


Figure 2.1: Collimated beam apparatus and radiometer. The collimated beam apparatus consists of a metal frame holding the UV lamp housing and two collimated tubes. The shutter (as indicated), to control irradiation exposure times, is shown in the closed position. A magnetic stirrer is shown with a petri dish placed under the left-hand collimated tube. The radiometer is on the right with a UV detector attached.

2.9 Determination of Photoreactivation in *Cryptosporidium* Oocysts

Duplicate samples containing 5×10^4 viable oocysts (Section 2.1.2.1) were irradiated using the germicidal wavelength of 254 nm (Section 2.8) with a dose of 1100 mJ/cm^2 , the UV dose confirmed as being cidal for oocysts according to the RT-PCR oocyst viability assay.

Following oocyst irradiation with the 254 nm wavelength of light, the oocysts were exposed to UV doses (ranging from 360 mJ/cm^2 to 1300 mJ/cm^2) at the 365 nm wavelength of light (Appendix 3.2) (the wavelength recognised as being the most effective at repairing UV-induced damage in other organisms) to test for the presence/absence of photoreactivation. For every UV dose tested at 365 nm, a negative control of 5×10^4 viable oocysts, which was exposed to the 254 nm wavelength of light but not exposed to the 365 nm wavelength was processed. A positive control, which was not exposed to either the 254 nm or 365 nm wavelength light, was also processed. To determine whether the 365 nm wavelength was detrimental to oocysts, a dose of 750 mJ/cm^2 was used.

CHAPTER 3 : RESULTS

At the outset, genetic information regarding *Cryptosporidium parvum* was required in order to establish an RT-PCR oocyst viability assay that uses the expression of the heat shock protein 70 (*hsp70*) gene to test the viability of *C. parvum* oocysts. For such a method, a number of practical factors had to be considered such as, ease of use, cost, speed, and avoidance of unnecessary manipulations to prevent contamination or degradation of mRNA. Two strains of *C. parvum* were used in this study; Camden (less commonly used in research work) and Iowa (commonly used in ultraviolet light studies: see Clancy *et al.*, 2004; Mofidi *et al.*, 2001; Shin *et al.*, 2001). Unless otherwise stated, all *C. parvum* oocysts used were of the Camden strain.

Previous studies (Clancy *et al.*, 2000; Drescher *et al.*, 2001; Shin *et al.*, 2001) have found ultraviolet (UV) light to be effective at killing *C. parvum* oocysts. In this study, an RT-PCR oocyst viability assay was established and used to determine the viability of oocysts in order to calculate the cidal UV dose for *C. parvum*. Once the cidal UV dose was determined, the process of photoreactivation was also investigated.

3.1 PCR Analysis of the *C. parvum* Strains Used in this Study

While the *C. parvum* Iowa strain was known to be of the bovine genotype (Waterborne™ Inc, New Orleans, USA & Clancy *et al.*, 2004), the genotype of the Camden strain was not known. To determine the genotype (*C. parvum* bovine vs. *C. hominis*) of oocysts of the Camden strain, restriction fragment length polymorphism (RFLP)-PCR analysis (Section 2.6.3) was used (Caccio *et al.*, 1999). Genomic DNA was isolated from Camden oocysts (Section 2.3.1) and used in a PCR (Section 2.5.2) with primers designed to amplify a 592 bp region of the *C. parvum* β -tubulin gene (TUB 1 & 2; see Table 2.1). The presence of the expected 592 bp PCR product was confirmed visually by agarose gel electrophoresis prior to digestion with *Dde*I and *Hae*III restriction enzymes in separate reactions (Figure 3.1, Gel A). The *C. parvum* bovine genotype β -tubulin sequence has a single *Dde*I endonuclease restriction site at nucleotide position 414 from the 5' end of the β -tubulin gene which

should give two digestion products of 414 bp and 178 bp and a single *HaeIII* restriction site at nucleotide position 380 from the 5' end which would generate two fragments of 380 bp and 212 bp. The corresponding *C. hominis* sequence would remain uncut with *DdeI* as the sequence lacks one of the restriction sites, but would produce two *HaeIII* digest products of 362 bp and 228 bp, as there is a restriction site at nucleotide position 362 from the 5' end. The two nucleotide difference seen with the combined *HaeIII* digestion product sizes generated for each genotype is due to a two base deletion within the intron sequence of the *C. hominis* genotype. Analysis of results from both enzyme digestion reactions, *DdeI* and *HaeII*, showed that the Camden strain was of the bovine genotype (Figure 3.1, Gel B) due to the presence of two correctly sized digest products with *DdeI* and with *HaeIII*.

To perform experiments on both *Cryptosporidium* strains (Camden and Iowa) in this study, it was necessary to establish that mRNA from both strains could be amplified by using the Cphsp 2423 & 2764 primer pair which are designed to amplify a 361 bp region of the *C. parvum hsp70* gene; the gene chosen for the optimised RT-PCR oocyst viability assay (see Figure 3.2 & Section 3.2.5.4). This was carried out in trial runs on mRNA extracted from both strains and the resulting 361 bp PCR products obtained were of the expected sizes. PCR products were then directly sequenced (Section 2.7.1) to confirm product specificity to the *C. parvum hsp70* gene. The sequences obtained from the 361 bp products were queried against the NCBI nucleotide database using the nucleotide-nucleotide BLASTN algorithm (Section 2.7.2). The top hit for each of the PCR products was to the *Cryptosporidium parvum* heat shock 70 kDa protein (*hsp70*) gene (accession number U11761) with the Camden strain returning an expect value of e-169 with 5 nucleotide mismatches present. The Iowa strain had an expect value of e-163 and 5 mismatches when compared with the same NCBI entry confirming that the PCR primers were specific for the *hsp70* gene in both *C. parvum* strains.

However, on some occasions a higher molecular weight band (with the apparent size of ~400 bp) was seen on the agarose gel in addition to the 361 bp band (Figure 3.2, Lanes 1 & 2). This artifact was sequenced from the Camden strain but the sequencing experiments only returned a total of 264 nucleotides from the 5' end. When this sequence was

compared to the NCBI database using BLASTN, the expect value against the accession number U11761 was e-141 with 2 nucleotide mismatches.

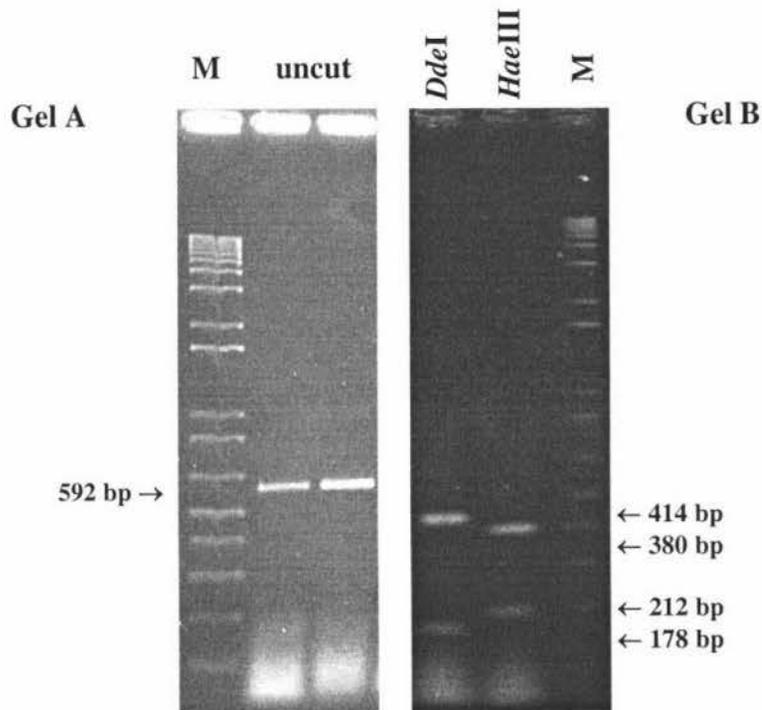


Figure 3.1: Gel A: Confirmation of the β -tubulin PCR product using primers TUB 1 & 2 prior to restriction digestion. Lane M: 1 Kb Plus DNA Ladder, Lanes 1 & 2: β -tubulin PCR products (592 bp) with *C. parvum* DNA (in duplicate). Gel B: RFLP-PCR analysis of Camden strain using *DdeI* and *HaeIII* restriction enzymes. Lane M: 1 Kb Plus DNA Ladder, Lane 1: PCR products (414 & 178 bp) from *DdeI* digestion, Lane 2: PCR products (380 & 212 bp) from *HaeIII* digestion.

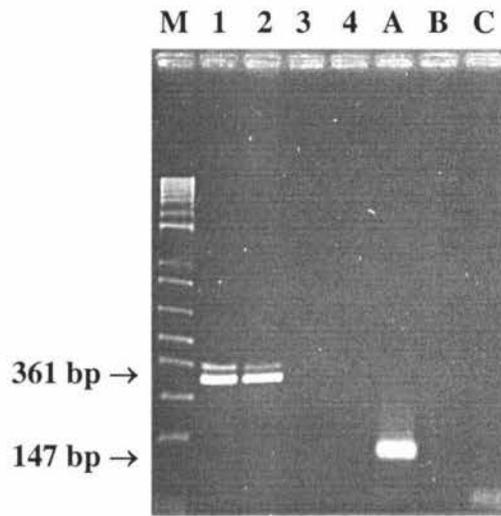


Figure 3.2: RT-PCR products using mRNA from *C. parvum* Camden and Iowa strains. Lane M: 1 Kb Plus DNA Ladder, Lane 1: Camden strain, Lane 2: Iowa strain, Lanes 3 & 4: Camden strain irradiated with a dose of 1000 mJ/cm² using 254 nm (in duplicate) Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control. For additional information regarding the UV irradiation data shown in Lanes 3 & 4 refer to Section 3.4.2.

3.2 Development of the RT-PCR Oocyst Viability Assay

Development of the RT-PCR oocyst viability assay required optimisation of several steps in the basic protocol (Stinear *et al.*, 1996) to ensure ease of use, rapid isolation of mRNA and increased sensitivity of oocyst detection without compromising the reliability of results. Table 3.1 lists the optimisation steps performed and the chosen procedures that are detailed in the following sections. For instance, *Cryptosporidium* oocysts are difficult to lyse and the nucleic acids released, contain DNA which was difficult to remove prior to using a combination of Dynabeads[®] Oligo (dT)₂₅ and DNaseI treatment (Section 3.2.4). For these reasons, a combination of components from a number of different commercial kits was used, as no one kit was successful by itself. The steps that were modified (Table 3.1) start from the origin of the oocysts and go all the way through to the type of buffer used during agarose gel electrophoresis when visualising the RT-PCR products.

3.2.1 Origin of *C. parvum* Oocysts

At the beginning of this study oocysts were extracted from locally collected faecal samples (Section 2.1.2.2), but it became clear that a new source of control oocysts, which were viable and available in large numbers was required. These oocysts needed to be imported from an outside supplier (Section 2.1.2.1) and both BTF Pty Ltd (North Ryde, NSW, Australia) and Waterborne[™] Inc. (New Orleans, USA) were chosen on the bases of affordability and dependability of supply.

3.2.2 Heat Shock Protein 70 (*hsp70*) Gene Expression

To increase oocyst detection sensitivity, an optimal expression level of heat shock mRNA was required (Section 2.2.3). In order to determine this, a set of assay trials with different heating times (1, 3, 12, 20, 30 minutes) at 45°C were conducted. From RT-PCR analysis detecting *hsp70* expression levels, it was determined the combination of 12 minutes at 45°C was optimal because it produced the strongest signal of expression with the shortest exposure time at 45°C as visualised in the agarose gel (result not shown).

Table 3.1: Optimisation stages of the RT-PCR oocyst viability assay

Step	Options	Method Option Chosen	Cross-Reference
Origin of oocysts	<ul style="list-style-type: none"> Extracted from faeces Imported from Australia 	Imported oocysts	Section 2.1.2
Heat shock of oocysts	<ul style="list-style-type: none"> Time exposed to 45°C (1, 3, 12, 20 and 30 minutes) 	12 minutes at 45°C	Section 2.2.3
Lysis of oocysts	<ul style="list-style-type: none"> Freeze/thaw Cell Lysis II Buffer 	Cell Lysis II Buffer for 20 minutes	Section 2.2.4
mRNA isolation & DNaseI treatment	<ul style="list-style-type: none"> mRNA beads* after freeze/thaw then DNaseI mRNA beads* after Cell Lysis II Buffer then DNaseI mRNA beads* after Cell Lysis II Buffer then DNA-free™ kit 	mRNA beads* after Cell Lysis II Buffer followed by the DNA-free™ kit	Section 2.2.4, 2.3.2 & 2.6.1
Type of RT-PCR	<ul style="list-style-type: none"> Two-step One-step 	One-step	Section 2.5.3
<i>Taq</i> DNA Polymerase used in RT-PCR	<ul style="list-style-type: none"> <i>Taq</i> DNA Polymerase (Qiagen) HotMaster™ <i>Taq</i> Polymerase (Eppendorf) 	HotMaster™ <i>Taq</i> Polymerase (Eppendorf)	Section 2.5.3
Primers used in RT-PCR	<ul style="list-style-type: none"> HSP FO/RO & FI/RI CHSP 1 & 4 Cphsp 2423 & 2764 	Cphsp 2423 & 2764	Table 2.1
RT-PCR cycle number	<ul style="list-style-type: none"> 35 to 44 cycles 	44 cycles	Section 2.5.3.1
Gel electrophoresis buffer	<ul style="list-style-type: none"> TAE buffer Sodium boric acid buffer 	Sodium boric acid buffer	Section 2.6.2

* mRNA beads refer to Dynabeads® Oligo (dT)₂₅ (DynaL Biotech)

3.2.3 Oocyst Lysis

Alternate freeze/thaw cycles, one minute each of using liquid nitrogen and boiling water, were originally used to lyse oocysts (Section 2.2.4.1). The Cell Lysis II Buffer (Ambion Inc.) was later chosen as the preferred method to lyse oocysts as it was quicker and just as effective. The use of Chelex[®] resin was no longer required as the results obtained with Cell Lysis II Buffer were similar both with and without inclusion of Chelex[®] (Figure 3.3, Lanes 2 & 3 and 9 & 10). There was also no difference in band intensity of downstream RT-PCR products between using 20 µl or 100 µl of Cell Lysis II Buffer (Figure 3.3, Lanes 2 & 4 and 9 & 11). Therefore, 20 µl of Cell Lysis II Buffer was chosen to conserve reagents.

In the modified protocol, proteinase K activity was removed by an additional 10 minute incubation at 75°C (to a total 20 minutes) as an extra step compared with the original manufacturers recommendations. This was necessary so that downstream reactions with DNaseI enzyme were not affected.

3.2.4 mRNA Isolation & DNaseI Treatment

Initially, mRNA was isolated by using Dynabeads[®] Oligo (dT)₂₅ (DynaL Biotech) (Section 2.3.2) from total oocyst nucleic acid extracted using freeze/thaw lysis (Section 2.2.4.1). This sequence of steps left genomic DNA still in the preparation, as indicated by the presence of a band in the agarose gel after electrophoresis of the RT-minus negative control reaction (data not shown). The method for oocyst lysis was therefore modified to using Cell Lysis II Buffer (Section 3.2.3) and followed by a DNaseI (Invitrogen and Roche) step to remove the residual genomic DNA. However, this combination of steps did not satisfactorily remove all DNA, so a further combination involving the majority of the above methods was trialled. Cell Lysis II Buffer was used to release total nucleic acid then the specific isolation of mRNA with Dynabeads[®] Oligo (dT)₂₅ (DynaL Biotech) followed by a DNaseI step to remove any contaminating DNA still present.

This final DNaseI step required optimisation by initially using a DNaseI supplied from Ambion Inc., instead of Invitrogen or Roche, with an increased amount of DNaseI (2U which is 3.3 times the amount recommended by Ambion Inc.) and increased incubation time (45 minutes instead of 30 minutes at 37°C). However, it was only through using the DNA-free™ kit (Ambion Inc.) (Section 2.6.1) which included a DNase Inactivation Reagent, instead of the Ambion DNaseI alone, could all traces of contaminating genomic DNA be removed. Confirmation of genomic DNA removal was inferred when no products were present in the RT-minus negative control (Figure 3.1).

3.2.5 Optimisation of RT-PCR Conditions for the Viability Assay

3.2.5.1 Comparison of One-step and Two-step RT-PCR Methods

Initially a two-step RT-PCR protocol was tested using commercial kits from Invitrogen, Roche and Ambion Inc with HSP FO/RO and HSP FI/RI, CHSP 1 & 4 and Cphsp 2423 & 2764 primer pairs tested with each kit (Section 2.5.3.2). Later, a one-step RT-PCR was adopted because it performed consistently, required less time and consumables, and produced good results (Section 2.5.3.1). Figure 3.3 shows a comparison between one- and two-step RT-PCR with the one-step RT-PCR clearly producing a greater yield of PCR product and therefore was thought to be of increased sensitivity compared with the two-step RT-PCR. Although both RT-minus negative controls seen in Figure 3.3, Lane B do show PCR product bands, indicating that some genomic DNA was present, these particular results were obtained prior to the inclusion of the DNaseI treatment and the main point to note is that the one-step RT-PCR protocol produced a greater yield than the two-step RT-PCR protocol.

3.2.5.2 Taq Polymerase Used in the RT-PCR

Preliminary trials with both one and two-step RT-PCR protocols using each of either *Taq* DNA Polymerase (Qiagen) or HotMaster™ *Taq* DNA Polymerase (Eppendorf) enzyme showed that HotMaster™ *Taq* DNA Polymerase performed better giving a greater yield of product (data not shown). As the one-step RT-PCR was ultimately chosen for the optimised RT-PCR oocyst viability assay and HotMaster™ *Taq* DNA Polymerase

performed well in both types of RT-PCR, the combination of a one-step RT-PCR using the HotMaster™ *Taq* DNA Polymerase was used.

3.2.5.3 RT-PCR Cycle Conditions

A number of cycling conditions for the one-step RT-PCR were trialled, these included: varying cycle number (35, 40, 42 or 44 cycles), increasing extension time from 30 seconds to 90 seconds and decreasing the extension temperature from 72°C to 65°C as this was the recommended temperature for optimal activity of HotMaster™ *Taq* DNA Polymerase (Eppendorf) (results not shown). As based on band intensity and specificity, results showed that 44 cycles and 90 seconds extension time at 65°C were optimal conditions for the one-step RT-PCR.

3.2.5.4 Primer Choice

Multiple primer pairs (Table 2.1) were trialled for both the one- and two-step RT-PCR protocols to test their reliability and level of sensitivity for *hsp70* mRNA detection. The positions of both the Cphsp 2423 & 2764 and CHSP 1 & 4 primer pairs within the *C. parvum hsp70* gene are shown in Figure 3.4, Part A. The reliability of the primers was important to consistently amplify mRNA from oocysts, as was the level at which oocysts could be detected for possible application of the RT-PCR oocyst viability assay to environmental water samples. For the two-step RT-PCR, an Oligo (dT)₁₈ was used in the reverse transcription followed by the HSP FO/RO and HSP FI/RI primer pairs (outers and inners of a nested PCR) in two sequential PCRs (data not shown). However, these nested primers were made redundant by the decision to use one-step RT-PCR for the optimised oocyst viability assay (Section 3.2.5.1). The first set of primers trialled in the one-step RT-PCR was the CHSP 1 & 4 primer pair. These primers appeared to degrade rapidly upon freezing which made them unreliable, although they were sensitive as they were able to detect down to 50 oocysts as seen in Figure 3.4, Part C, Lane 5. For example, out of 9 times that 5×10^4 oocysts were used as a positive control only 4 times (44%) did the CHSP 1 & 4 primer pair give a positive result.

The Cphsp2423 & 2764 primer pair was chosen over the CHSP 1 & 4 primer pair for the optimised method, as they were reliable and sensitive. The sensitivity was increased through optimisation of the RT-PCR cycle number (Section 3.2.5.3); an oocyst detection sensitivity of 200 oocysts was achieved with 40 cycles and 100 oocysts with 44 cycles (results not shown). This was further increased to a detection limit of 50 oocysts by using 44 cycles of the RT-PCR programme and increasing the HotMaster™ *Taq* Polymerase (Eppendorf) from 2 U to 5 U (Figure 3.4, Part B, Lane 3). The amount of *Taq* Polymerase was later reduced to 4 U as this gave clearer band visualisation in the agarose gel.

3.2.6 Comparison Between Two Electrophoresis Buffers for Visualisation of RT-PCR Products

In order to visualise results for the RT-PCR oocyst viability assay, agarose gel electrophoresis was used. It was therefore important that good resolution was shown in the agarose gel and that electrophoresis was quick to complete for timely results. Both TAE and sodium boric acid based buffers (Appendix 1.1 & 1.2) were used separately for gel electrophoresis in trials to determine which gave the clearest results in the fastest time. As shown in Figure 3.5 (Gels A and B), both buffers showed good resolution of the RT-PCR product bands, however, gel electrophoresis (Section 2.6.2) was quicker when using the sodium boric acid buffer based gel at 20 minutes than when using the TAE buffer based gel at 120 minutes. For these reasons, a 1.5% agarose gel with sodium boric acid buffer was used to visualise RT-PCR products from the optimised oocyst viability assay. Electrophoresis was reduced from 350 V (Figure 3.5, Gel A) to 300 V as this gave better separation of the bands in the 1 Kb Plus DNA ladder (data not shown).

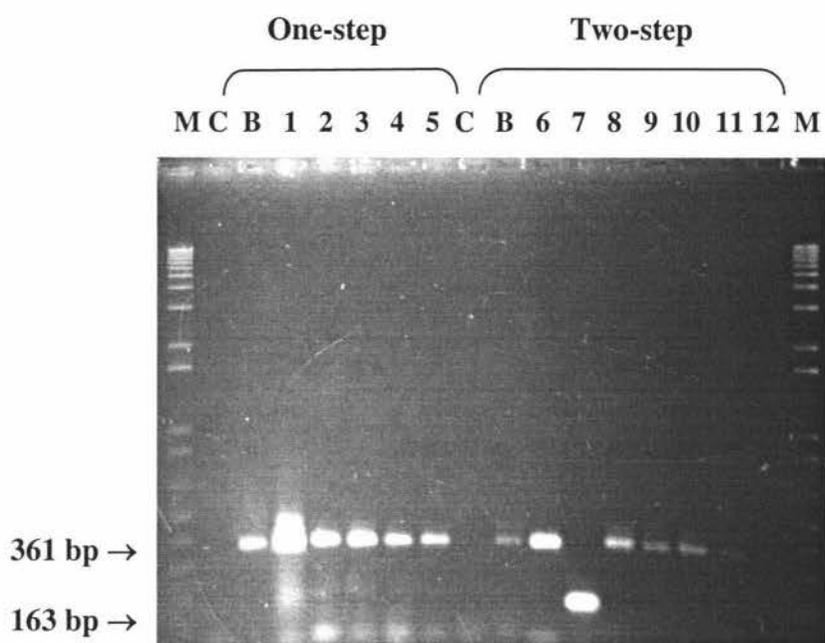


Figure 3.3: Comparison of one- and two-step RT-PCR. Lane M: 1 Kb Plus DNA Ladder, Lane C: minus-template negative control, Lane B: RT-minus negative control lanes 1 & 6: Ambion kit (RT-PCR) positive control. Lanes C to 5 show one-step RT-PCR products made from oocysts undergoing the following described conditions, Lanes C to 12 show two-step RT-PCR products made from oocysts undergoing the following described conditions. RT-PCR was conducted using the Cphsp 2423 & 2764 primer pair (for the two-step RT-PCR, the RT stage used Oligo (dT)₁₈ primer). Lane 1: Ambion kit (RT-PCR) positive control, Lane 2: 5×10^4 oocysts with Chelex[®] and 100 μ l Cell Lysis II Buffer, Lane 3: 5×10^4 oocysts without Chelex[®] and 100 μ l Cell Lysis II Buffer, Lane 4: 5×10^4 oocysts with Chelex[®] and 20 μ l Cell Lysis II Buffer, Lane 5: 5×10^4 oocysts without Chelex[®] and 20 μ l Cell Lysis II Buffer, Lane 6: Ambion kit (RT-PCR) positive control, Lane 7: *Giardia* trophozoite DNA as a positive control, Lane 8: 5×10^4 oocysts lysed with 100 μ l Cell Lysis II Buffer and without a DNaseI treatment, Lane 9: 5×10^4 oocysts with Chelex[®] and 100 μ l Cell Lysis II Buffer, Lane 10: 5×10^4 oocysts without Chelex[®] and 100 μ l Cell Lysis II Buffer, Lane 11: 5×10^4 oocysts with Chelex[®] and 20 μ l Cell Lysis II Buffer, Lane 12: 5×10^4 oocysts without Chelex[®] and 20 μ l Cell Lysis II Buffer.

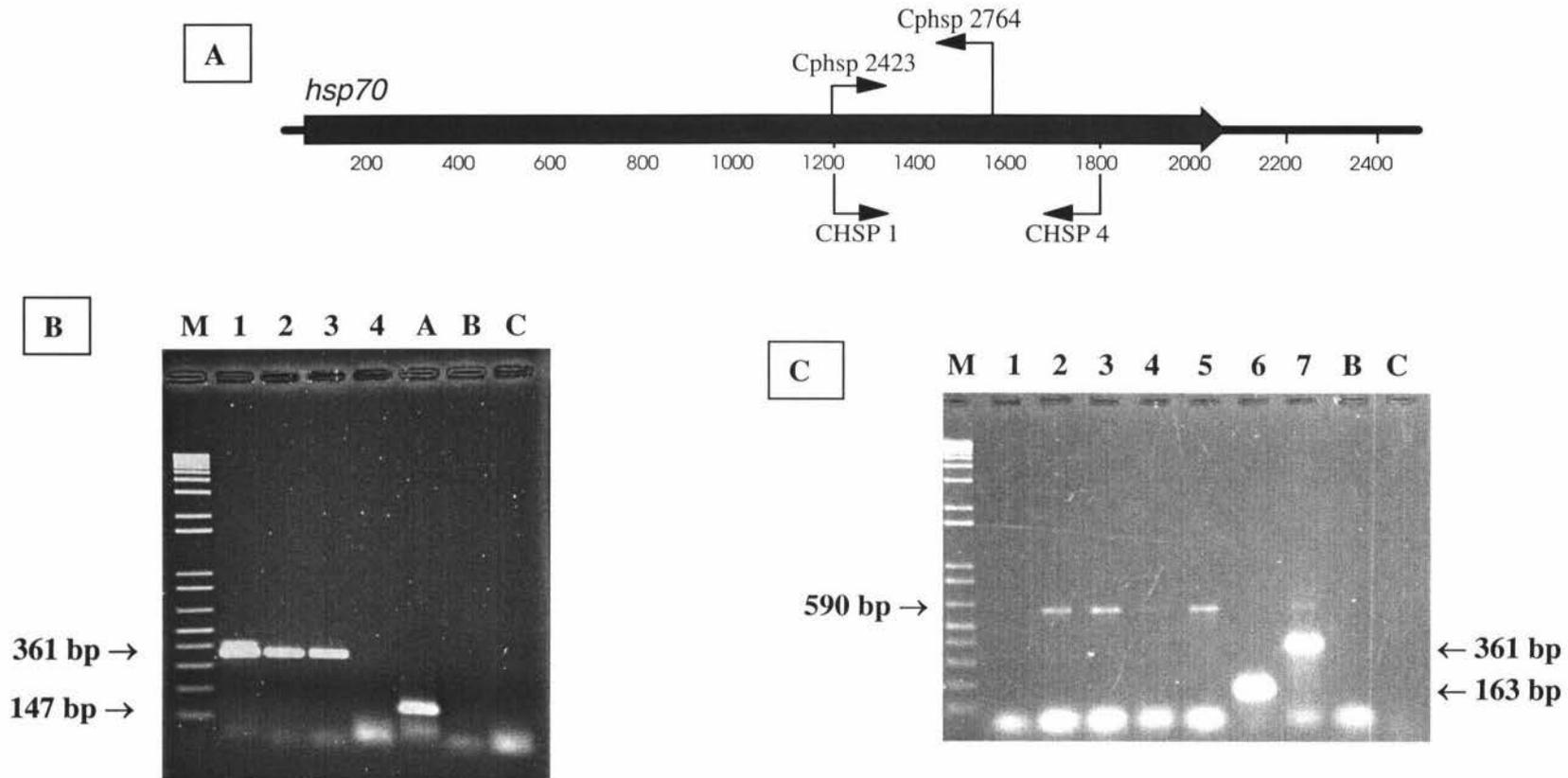


Figure 3.4: RT-PCR primer sensitivities and primer position on the *hsp70* gene. **A:** Schematic diagram of *hsp70* gene (GenBank accession number U11761) showing the location of primer pairs Cphsp 2423 & 2764 and CHSP 1 & 4. **B:** One-step RT-PCR using Cphsp 2423 & 2764 primer pair. Lane M: 1 Kb Plus DNA Ladder, Lane 1: 5×10^4 oocysts, Lane 2: 100 oocysts, Lane 3: 50 oocysts, Lane 4: 10 oocysts, Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control. **C:** One-step RT-PCR using CHSP 1 & 4 primer pair. Lane M: 1 Kb Plus DNA Ladder, Lane 1: 5×10^4 oocysts, Lanes 2 & 3: 100 oocysts (in duplicate), Lanes 4 & 5: 50 oocysts (in duplicate), Lane 6: *Giardia* trophozoite DNA as a positive control, Lane 7: Ambion kit (RT-PCR) positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

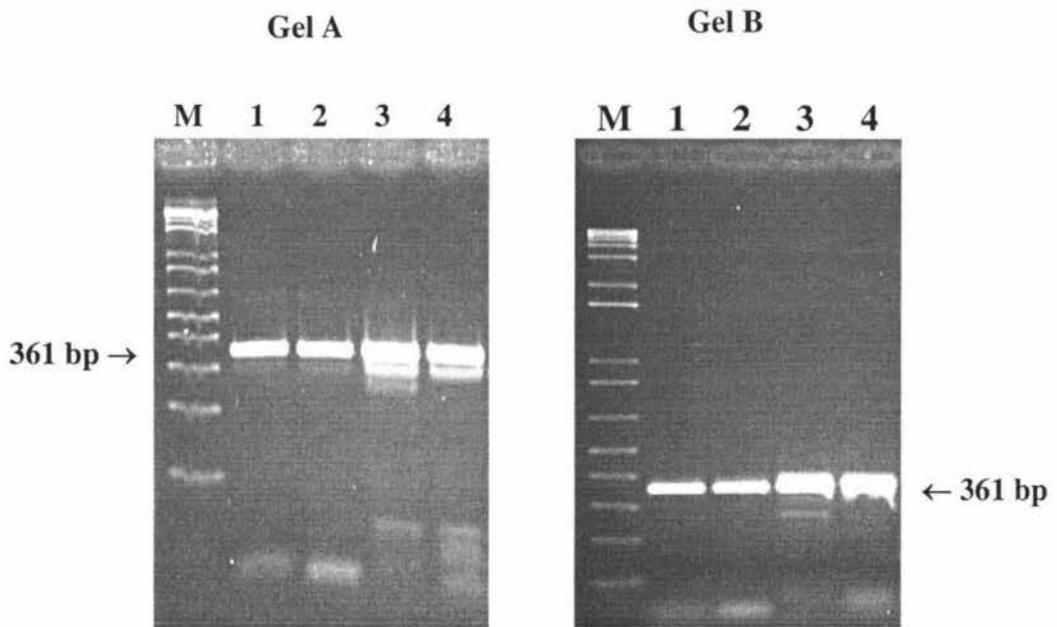


Figure 3.5: Comparison between using sodium boric acid buffer and TAE buffer for agarose gel electrophoresis.

Gel A: 2% sodium boric acid buffer based agarose gel, run at 350 V for approximately 20 minutes. Lane M: 1 Kb Plus DNA Ladder, Lanes 1 & 2: sample 1 (in duplicate), Lanes 3 & 4: sample 2 (in duplicate).

Gel B: 2% TAE buffer based agarose gel, run at 80 V for 2 hours. Lane M: 1 Kb Plus DNA Ladder, Lanes 1 & 2: sample 1 (in duplicate), Lanes 3 & 4: sample 2 (in duplicate).

3.3 Ultraviolet Light Experiments

To determine a cidal UV dose for *C. parvum* oocysts using the RT-PCR oocyst viability assay, a number of technical factors had to be considered. These are detailed in the sections below. As oocysts needed to be suspended in liquid during UV irradiation, petri dishes were used (Section 2.8). Due to the larger volume that oocysts were isolated from (compared to the method used in Section 2.2.2), oocyst detection sensitivity was re-tested and the level of irradiance emitted from the UV lamps was also checked for consistency. Prior to choosing either 1x PBS or DEPC-treated water to suspend oocysts in, turbidity readings were performed to ensure that turbidity would not affect the determination of a cidal UV dose (Appendix 2).

3.3.1 Turbidity Readings

Turbidity readings were measured (Appendix 2) to determine whether the UV absorbed by oocysts suspended in either 1x PBS (Appendix 1.5) or DEPC-treated water (Section 2.4.2) was affected by turbidity. As seen in Table 3.2, the DEPC-treated water was measured at an average of 0.07 NTU and as this was closest to 0.1 NTU, which is the water quality standard required of water treatment plants (Ministry of Health New Zealand, 2000), DEPC-treated water was used to suspend oocysts in the petri dish when determining a cidal UV dose (Section 2.8) instead of 1x PBS.

Table 3.2: Turbidity readings of 1x PBS and DEPC-treated water

	Readings from this study (NTU ¹)	CEL ² readings (NTU ¹)
1x PBS	1.0 and 1.38 (Average = 1.19)	0.9
DEPC-treated water	0.05 and 0.09 (Average = 0.07)	0.05

¹ Nephelometric Turbidity Units (NTU)

² Central Environment Laboratory (CEL)

3.3.2 RT-PCR Oocyst Detection Sensitivity when Isolated from a Petri Dish

For UV irradiation, oocysts were resuspended in 10 mL DEPC-treated water (Section 2.8). A reduction in the petri dish volume (10 mL) to a tube volume (100 µl), ready for the heat shock process (Section 2.2.3) prior to the RT-PCR, was therefore required. The procedure for trials during the development of the RT-PCR oocyst viability assay (Section 3.2.5.4) used oocysts that were isolated from PCR tubes (Section 2.2.2) instead of from petri dishes. Therefore it was necessary to determine the number of oocysts that the RT-PCR could detect down to when oocysts had been isolated from a petri dish. Two batches of oocysts, both of *C. parvum* (Camden strain), were used to do this.

Inconsistent levels of oocyst detection sensitivity by the RT-PCR procedure were found when oocysts were isolated from a petri dish as seen in Table 3.3. As expected, the percentage of times that oocysts were detected for a small number of oocysts was low and this increased with an increase in oocyst number (Table 3.3). Unexpectedly, the level of oocyst detection when the oocyst number ranged from 200 to 800 oocysts was very low (ranging for 0% to 33%). Also, these trials showed a larger quantity of oocysts was required (between 900 and 5×10^3) for reliable oocyst detection when oocysts were isolated from a petri dish (Table 3.3) than when isolated from a PCR tube as in trials for the development of the RT-PCR oocyst viability assay (Section 3.2.5.4) which had an oocyst detection limit of 50 oocysts.

3.3.3 Consistency of UV Irradiance Emitted from Collimated Tubes

Two collimated tubes were used to direct UV light onto the oocyst suspension during UV irradiation (Section 2.8). A continual reading of UV irradiance could not be made, as the detector attached to the radiometer could not be placed under the collimated tube simultaneously with the petri dish during UV exposure. Therefore, irradiance readings were taken pre- and post-UV irradiation to ensure that during the exposure time a consistent level of UV was emitted from the UV lamp (Section 2.8.2). As the level of UV irradiance emitted could differ between tidal UV dose experiments, readings were taken each time.

However, the two collimated tubes showed consistency in the amount of UV irradiance emitted as seen in Table 3.4.

The pre-UV exposure reading was taken so an exposure time could be calculated for the UV dose required. For example, using values from Table 3.4 and the equation in Appendix 4.2, to calculate an 1100 mJ/cm^2 dose, if the pre-UV exposure irradiance reading was 0.24 mW/cm^2 , then the UV dose required (1100 mJ/cm^2) would be divided by this. Therefore, the exposure time required would be 4489 seconds (or 74 minutes 49 seconds). Using the post-UV exposure reading and in conjunction with the exposure time of UV for the oocysts already given, an overall average UV dose would be calculated. For this example, the post-UV reading (0.25 mW/cm^2) would be multiplied by the exposure time (4489 seconds), equalling 1122 mJ/cm^2 . Therefore, the overall average UV dose given is the average of 1100 and 1122 mJ/cm^2 , which is 1111 mJ/cm^2 .

Table 3.3: Oocyst detection sensitivity of RT-PCR when oocysts are isolated from a petri dish

Number of oocysts	Number of samples tested	Percentage of times detected
First batch of oocysts		
80	4	25
100	4	50
200	3	33
400	1	0
800	2	0
900	3	100
1000	4	75
Second batch of oocysts		
5	2	0
50	2	0
500	2	0
5000	2	100

Table 3.4: Average UV irradiance pre- and post-UV exposure

Reading	mW/cm ²	Average reading (mW/cm ²)
Pre-UV exposure	0.24	0.245
Post-UV exposure	0.25	

3.3.4 Cidal UV Dose Determination for *C. parvum* Oocysts

The purpose of determining the cidal UV dose was so that it could be used to kill oocysts in experiments which tested for the process of photoreactivation (Section 3.3.5). All samples used in trials to determine the cidal UV dose contained 5×10^4 viable *C. parvum* oocysts as, this number of oocysts could be detected with the RT-PCR oocyst viability assay when a cidal UV dose was not given and this helped prevent false negative results. The oocysts were irradiated with UV light at a wavelength of 254 nm, with doses ranging between 10 mJ/cm² and 1100 mJ/cm² (Section 2.8). The original low dose of 10 mJ/cm² was chosen to replicate other previously conducted research (Bukhari *et al.*, 1999; Clancy *et al.*, 2000) with the point of difference being the method of testing viability (i.e. using the RT-PCR oocyst viability assay rather than animal infectivity). A non-irradiated sample was used as a positive control (Figure 3.6, Lane 1) to confirm that the oocysts were viable prior to UV irradiation.

Surprisingly, in contrast to the previous studies (Bukhari *et al.*, 1999; Clancy *et al.*, 2000), the low UV doses (10 and 20 mJ/cm²) did not kill the oocysts as determined by the RT-PCR oocyst viability assay; therefore higher doses of UV were tested. Expression of the *hsp70* gene was detected at doses up to and including 500 mJ/cm², indicating that these doses did not kill oocysts. However, half of the trials using 750 mJ/cm² proved cidal for oocysts (Figure 3.6, Lanes 6 & 7 and Table 3.5). More reliably, 1000 and 1100 mJ/cm² were shown to be cidal for oocysts in 100% of the tests performed (Figure 3.2, Lanes 3 & 4 and Figure 3.6, Lanes 8 & 9 and Table 3.5). To find a consistently lower cidal UV dose between 750 and 1100 mJ/cm², smaller increments in the UV dose given to oocysts would need to be performed.

Clancy *et al.*, 2004 showed that there is little difference in UV susceptibility between five different strains of *C. parvum*, however, Camden was not included in their study. Therefore, to determine whether there was a difference in UV susceptibility between the Camden and Iowa strains, a dose of 350 mJ/cm² was given to Iowa oocysts (Figure 3.7); a dose that was not cidal for Camden oocysts (Figure 3.6). One trial of a duplicate sample of

Iowa oocysts was tested with 350 mJ/cm² and this was not found to be cidal in either sample. Due to time restraints, testing with other UV doses was not undertaken with Iowa oocysts.

A different gene (β -tubulin), other than the *hsp70* gene, was also used as a viability marker with RT-PCR to test for the cidal UV dose of *C. parvum* Camden oocysts. Doses of 40, 200 and 400 mJ/cm² were tested using β -tubulin primers, TUB 1 & 2 (Table 2.1), with all doses found to be cidal (results not shown). This contrasts with results using the *hsp70* gene as a genetic marker of viability (Figure 3.6). In another trial monitoring the expression of the β -tubulin gene, doses of 10, 20 and 40 mJ/cm² were found to be cidal with an anomaly at 30 mJ/cm² (Figure 3.8, Lane 4). Interestingly, these low cidal UV doses are more consistent with data found in research conducted using mice infectivity to determine oocyst viability (Bukhari *et al.*, 1999; Clancy *et al.*, 2000).

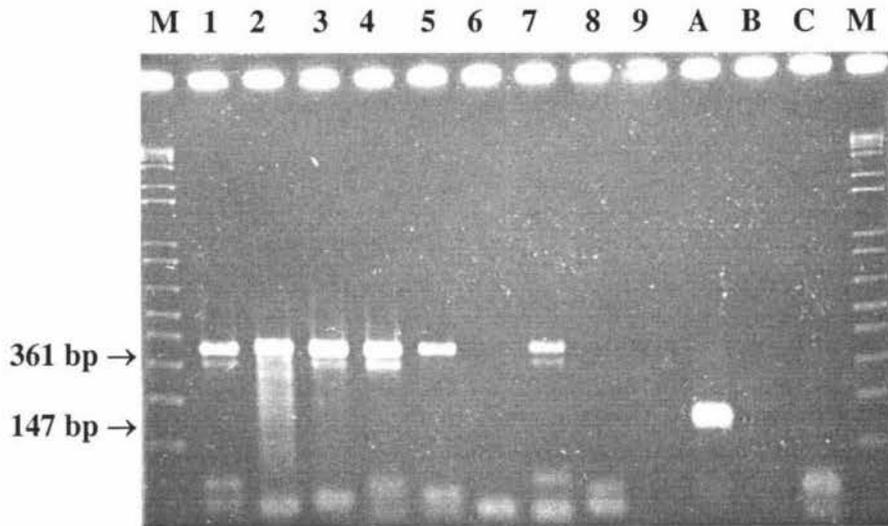


Figure 3.6: Cidal UV dose determination for Camden oocysts using Cphsp 2423 & 2764 primer pair. Lane M: 1 Kb Plus DNA Ladder, Lane 1: non-irradiated sample, Lane 2: oocysts irradiated with 10 mJ/cm², Lane 3: oocysts irradiated with 20 mJ/cm², Lane 4: oocysts irradiated with 350 mJ/cm², Lane 5: oocysts irradiated with 500 mJ/cm², Lanes 6 & 7: oocysts irradiated with 750 mJ/cm² (in duplicate), Lane 8: oocysts irradiated with 1000 mJ/cm², Lane 9: oocysts irradiated with 1100 mJ/cm², Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

Table 3.5: Cidal UV dose determination trials

Dose of UV light (mJ/cm ²)	Number of samples tested	Number of times UV dose was cidal
10	2	0
20	2	0
350	5	0
500	4	0
750	2	1 (50%)
1000	4	4 (100%)
1100	9	9 (100%)

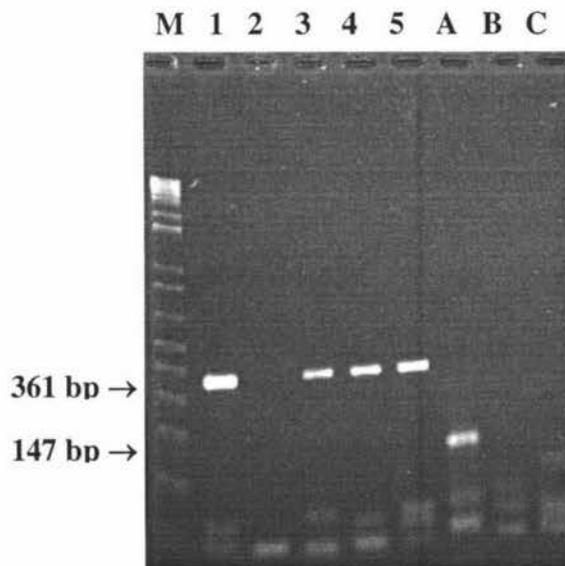


Figure 3.7: Effect of a 350 mJ/cm² UV dose on oocysts of Camden and Iowa strains. Lane M: 1 Kb Plus DNA Ladder, Lane 1: non-irradiated Camden strain, Lanes 2 & 3: Camden strain irradiated with 750 mJ/cm² (in duplicate), Lanes 4 & 5: Iowa strain irradiated with 350 mJ/cm² (in duplicate), Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

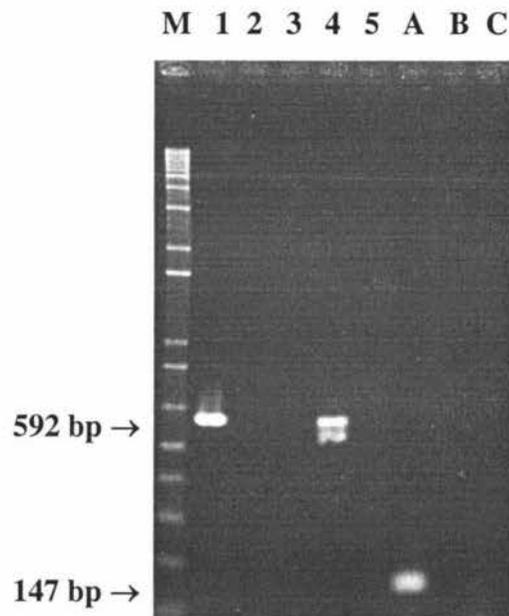


Figure 3.8: Cidal UV dose determination for Camden oocysts using β -tubulin primers. Lane M: 1 Kb Plus DNA Ladder, Lane 1: non-irradiated oocysts, Lane 2: oocysts irradiated with 10 mJ/cm^2 , Lane 3: oocysts irradiated with 20 mJ/cm^2 , Lane 4: oocysts irradiated with 30 mJ/cm^2 , Lane 5: oocysts irradiated with 40 mJ/cm^2 , Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

3.3.5 Determination of Photoreactivation in *C. parvum* Oocysts

To test for the presence/absence of the process of photoreactivation, oocysts were given a cidal UV dose of 1100 mJ/cm^2 (as determined in Section 3.3.4) using a wavelength of 254 nm (Section 2.8), prior to exposure to 365 nm wavelength UV light. Previous studies investigating the process of photoreactivation in *C. parvum* (Oguma *et al.*, 2001; Shin *et al.*, 2001) used UV light at the 365 nm wavelength to determine whether oocysts could undergo internal genetic repair enabling oocysts to function after being killed with a cidal UV dose. Therefore, tests were firstly conducted to check whether UV light at the 365 nm wavelength was detrimental to oocysts and to ensure that this wavelength was not the cause

of any future apparent absence of photoreactivation. A dose of 750 mJ/cm² was chosen to irradiate oocysts for these trials as it was in the middle of the range of UV doses used in the photoreactivation trials (Section 2.9). The same level of *hsp70* gene expression was present both with and without irradiation from the 365 nm wavelength UV light (results not shown), indicating no detrimental effects occur with 365 nm wavelength irradiation.

The UV doses (between 360 and 1300 mJ/cm²) at the 365 nm wavelength were chosen to replicate conditions from previous research (Oguma *et al.*, 2001; Shin *et al.*, 2001) (Table 3.6). The increments at which each dose increased was originally set at approximately double the previous dose with the intention that had there been an indication of the presence of photoreactivation, doses in between these values would be tested (as was done with the 800 mJ/cm² dose). At these UV doses, no photoreactivation was detected in *C. parvum* oocysts when using *hsp70* gene expression and the RT-PCR oocyst viability assay as a viability test.

Table 3.6: Determination of photoreactivation in oocysts

Dose of 365 nm UV light (mJ/cm ²)	Number of times tested	Number of times oocysts photoreactivated
360	2	0
650	2	0
800	2	0
1300	2	0

3.4 Effect of Heat Shock and Heat Inactivation Conditions

The optimal level of *hsp70* mRNA expression was achieved by using heat shock conditions (12 minutes at 45°C, see Section 3.2.2) on *C. parvum* oocysts, resulting in increased oocyst detection sensitivity of the RT-PCR oocyst viability assay. Further experiments could then be carried out to confirm the findings of Stinear *et al*, (1996) that the inclusion or exclusion of the heat shock step had no effect on the level of *hsp70* mRNA detectable when using RT-PCR. This was done by exposing oocysts either to heat shock or not, extracting mRNA from the oocysts and using this in RT-PCR tests (Section 2.5.3.1). A visually identical level of *hsp70* mRNA was detected both with and without heat shock conditions. However, it should be noted that the RT-PCR oocyst viability assay is not quantitative and this conclusion is strictly based on a visual comparison of the products in the agarose gel (Figure 3.9).

In addition to heat shock experiments, heat inactivation (20 minutes at 100°C) trials were investigated as Hallier-Soulier and Guillot, (2003) found that these conditions were sufficient to kill oocysts and that *hsp70* mRNA was not detected afterwards. However, when oocysts were exposed to these conditions in the present study a visually identical level of *hsp70* mRNA was detected as with oocysts which had been exposed to heat shock conditions (Figure 3.9).

A different genetic marker, β -tubulin, was also investigated using the TUB 1 & 2 primer pair (Table 2.1) to examine whether this would show different results to *hsp70* under the previously defined heat shock and heat inactivation conditions (Figure 3.10). In these RT-PCR tests there was production of β -tubulin mRNA under heat shock conditions, but with heat inactivation the β -tubulin mRNA was no longer detectable. These β -tubulin results (Figure 3.10, Lanes 3 & 4) are in contrast to the ones produced using the *hsp70* gene as a marker (Figure 3.9, Lanes 1 & 3). Two bands (~ 250 & 370 bp) in addition to the expected β -tubulin RT-PCR product (592 bp) can be seen in Figure 3.10, Lane 3 and are from non-specific binding. These may have been caused by the fact that the RT-PCR programme

(Section 2.5.3.1) used was optimised for the Cphsp 2423 & 2764 primer pair and not for the TUB 1 & 2 primer pair (Table 2.1).

The stability of *hsp70* mRNA within oocysts was also investigated as this may have an effect on the interpretation of results. Thus, if mRNA persists within dead oocysts, under some circumstances such as with a high number of oocysts, false positive viability determination may occur. Unlike previous investigators (Baeumner *et al.*, 2001; Morgan and Thompson, 1998; Stinear *et al.*, 1996), who observed that mRNA was not detectable from dead oocysts, tests conducted here using the optimised RT-PCR oocyst viability assay found that post-heat inactivated oocysts held at room temperature for 70 hours still contained detectable *hsp70* mRNA (Figure 3.11).

The amount of *hsp70* mRNA present in a sample of 5×10^4 resting un-heat shocked oocysts is likely to be high and may account for finding no observable difference in *hsp70* gene expression with and without heat shock conditions (Figure 3.9). Therefore, samples with lower numbers of oocysts were tested to see whether *hsp70* mRNA was still detectable, both with and without heat shock, using RT-PCR. No observable difference in *hsp70* gene expression with and without heat shock was seen with oocyst numbers as low as 3000 (Table 3.7). The negative results obtained under both experimental conditions with 1000 oocysts may be because this number of oocysts falls below the detection limit of the RT-PCR oocyst viability assay when oocysts are isolated from a petri dish (Table 3.3).

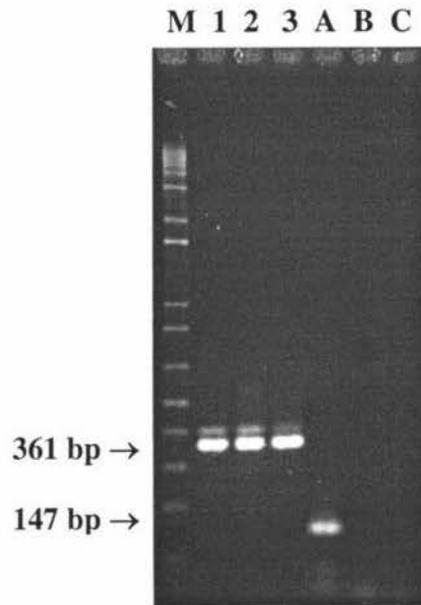


Figure 3.9: Effect of heat shock and heat inactivation conditions on *hsp70* mRNA levels. Lane M: 1 Kb Plus DNA Ladder, Lane 1: *hsp70* RT-PCR product from oocysts exposed to heat shock conditions, Lane 2: *hsp70* RT-PCR product from oocysts not exposed to heat shock conditions, Lane 3: *hsp70* RT-PCR product from oocysts exposed to heat inactivation conditions, Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

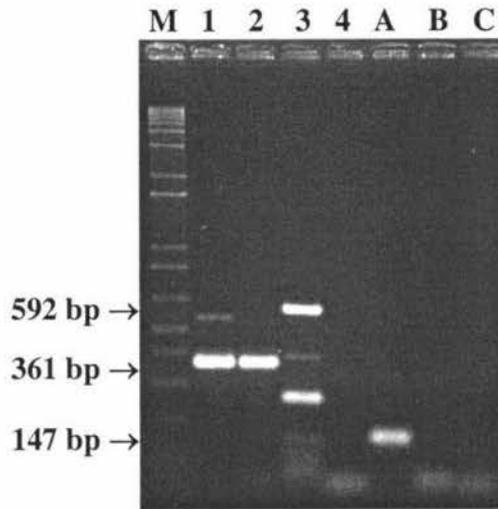


Figure 3.10: Effect of heat shock and heat inactivation conditions on *hsp70* and β -tubulin mRNA levels. Lane M: 1 Kb Plus DNA Ladder, Lane 1: *hsp70* RT-PCR product from oocysts exposed to heat shock conditions, Lane 2: *hsp70* RT-PCR product from oocysts exposed to heat inactivation conditions, Lane 3: β -tubulin RT-PCR products from oocysts exposed to heat shock conditions, Lane 4: β -tubulin RT-PCR product from oocysts exposed to heat inactivation conditions, Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

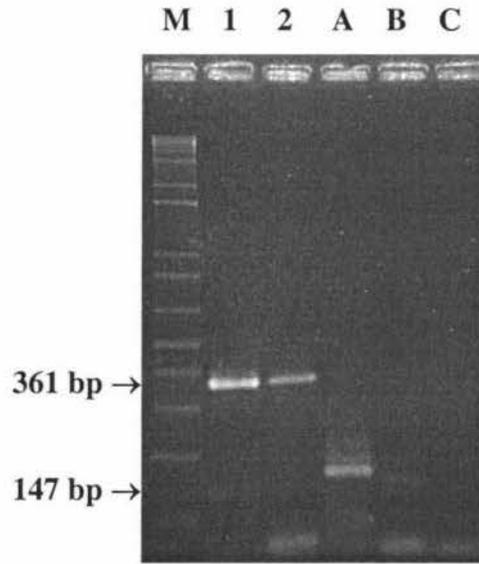


Figure 3.11: Stability of *hsp70* mRNA within oocysts held at room temperature, 70 hours post-heat inactivation. Lane M: 1 Kb Plus DNA Ladder, Lane 1: *hsp70* RT-PCR product from oocysts exposed to heat shock conditions, Lane 2: *hsp70* RT-PCR product from oocysts exposed to heat inactivation conditions followed by 70 hours at room temperature then heat shock conditions, Lane A: Armoured RNA[®] positive control, Lane B: RT-minus negative control, Lane C: minus-template negative control.

Table 3.7: Detection of *hsp70* mRNA with and without heat shock conditions with varying oocyst number

Number of oocysts	Number of times trialled with & without heat shock	mRNA detected with heat shock	mRNA detected without heat shock
5x10 ⁴	3	+	+
5000	2	+	+
3000	2	+	+
1000	2	-	-

+ indicates that *hsp70* mRNA was detected using RT-PCR

- indicates that *hsp70* mRNA was not detected using RT-PCR

CHAPTER 4 : DISCUSSION

The major objective for this project (Section 1.13) is to develop an RT-PCR oocyst viability assay, which is reliable and sensitive. The results presented in Section 3.2 show that this has been successfully achieved but only after considerable work to modify parts of an existing protocol (Stinear *et al.*, 1996). This included such steps as oocyst lysis right through to the type of RT-PCR methodology chosen and the protocol conditions and reagents which gave optimal results. A series of secondary objectives were also completed including using this improved RT-PCR assay to calculate a tidal UV dose for *C. parvum* oocysts. The general utility of the new RT-PCR oocyst viability assay as a testing method was compared to previous UV studies which employed methods such as animal and cell culture infectivity (Bukhari *et al.*, 1999; Clancy *et al.*, 2004; Drescher *et al.*, 2001; Shin *et al.*, 2001). The phenomenon of photoreactivation of UV killed oocysts was investigated and experiments on the effects of heat inactivation on *hsp70* and β -tubulin mRNA levels were also carried out.

4.1 Difficulties with Using *Cryptosporidium* as an Experimental Organism

Live *Cryptosporidium* is not easily cultured under laboratory conditions due to the complex nature of the lifecycle and so for this study they were purchased as oocysts from an Australian supplier (BTF Pty Ltd.) and imported into New Zealand under a Ministry of Agriculture and Forestry permit. Therefore, supplies of *Cryptosporidium* oocysts were limited due in part to economic reasons. The total cost of 1×10^7 oocysts, including a shipping and dangerous goods handling fee because the oocysts were viable, was AUS\$1200. Consequently, when one must deal with relatively small quantities, typically 1×10^7 oocysts, problems arise for laboratory procedures because a limited range and number of experiments can be conducted and a limited amount of genetic material is available.

4.2 PCR Analysis of the *C. parvum* Strains Used in this Study

A short characteristic target region was sequenced from both strains (Camden and Iowa) used in this study to determine if differences existed which might have consequences for the use of the Cphsp 2324 & 2764 primer pair in the RT-PCR of *hsp70* mRNA (Section 2.5.3.1). The two sequences were found to be very similar to one another (Section 3.1). This eliminated the possibility of false negative results due to the primers in the RT-PCR not amplifying mRNA from a particular strain. Both strains were shown to be of the bovine genotype (Section 3.1). This is an important finding, as this could have influenced the results of UV susceptibility tests since each strain has been shown to have differential susceptibility by Clancy *et al.* (2004).

4.3 Development of the RT-PCR Oocyst Viability Assay

RT-PCR was chosen as a method on which to base a viability assay on the premise that live organisms must carry out transcription and so produce detectable mRNA (Hallier-Soulier and Guillot, 2003; Morgan and Thompson, 1998; Stinear *et al.*, 1996). An oocyst viability assay based on the detection of *hsp70* mRNA expression was selected to overcome fairly obvious shortcomings inherent in other viability methods used for previous studies, as detailed in Section 1.10.2.3. The *hsp70* gene was chosen as a reliable target for a viability marker because under conditions of stress, *hsp70* is the major cellular product of protein synthesis. There is a 1000 to 10 000-fold increase in its mRNA under these conditions, ensuring a sufficient amount of the target to permit sensitive detection by the RT-PCR oocyst viability assay (Stinear *et al.*, 1996). Many researchers have suggested that *hsp70* mRNA is unstable at room temperature. Thus it has a short post mortem half-life and is unlikely to be preserved in dead oocysts under ambient conditions (Hallier-Soulier and Guillot, 2003; Stinear *et al.*, 1996).

A basic assay protocol (Stinear *et al.*, 1996) was used in preliminary trials followed by optimisation of as many steps in the protocol as were required to render the modified assay effective for the desired applications. The leading aims of the assay development work

were to make the procedure quick and reliable. Speed is important for two reasons; first to prevent degradation of mRNA and second so that results could be available quickly. This is important for example, to help clients to put steps in place to stop viable oocysts from getting out of a water treatment plant. The reliability of the assay for determining viability of oocysts is an equally important consideration. However, it was recognised that if any false results were to be tolerated it would be much better, with respect to public health concerns, to return a false positive than a false negative.

In order to make the RT-PCR oocyst viability assay most effective, elements from several commercial kits were utilised, as no one kit alone was sufficient (Section 3.2). The first stage of the development process was to ensure that a dependable supply of oocysts for use in trials were consistently viable (Section 2.1.2.1). This introduced an element of constancy and confidence versus uncertainty surrounding viability of oocysts extracted from faeces as used in preliminary trials (Section 2.1.2.2). This also saved preparation time, as extraction and purification of oocysts from biological sources were no longer required as steps in the procedure.

Next, the conditions required to express the *hsp70* gene at its optimal level for the assay were determined (Section 3.2.2). The shortest time at 45°C needed to give the strongest signal of *hsp70* gene expression, as shown by electrophoresis of the RT-PCR product in the agarose gel, was found to be 12 minutes (Section 3.2.2). This time was judged to be optimal. Hallier-Soulier and Guillot (2003) found that when oocysts were initially frozen at -80°C then thawed at 4°C, prior to heating at 42°C, the RT-PCR signal obtained in the assay was intensified. However, when this procedure was replicated in the present study, it did not appear to increase the amount of RT-PCR product obtained and therefore these conditions were rejected for the optimised RT-PCR oocyst viability assay (Section 3.2).

Cryptosporidium oocysts are notoriously difficult to lyse. However, Cell Lysis II Buffer (Ambion Inc.) chosen for the optimised RT-PCR oocyst viability assay was found to be effective. It also inactivates endogenous RNases thereby preventing degradation of cellular RNA (Ambion Inc, 2004). The original incubation time of 10 minutes at 75°C, as

recommended by Ambion Inc., was increased to 20 minutes as this helped prevent downstream reactions with DNaseI from being compromised by residual proteinase K activity in the Cell Lysis II Buffer (Section 3.2.3).

High quality mRNA, with all genomic DNA and all RNases removed, was essential for use in the RT-PCR assay. Contaminating *Cryptosporidium* DNA proved difficult to remove from the mRNA preparations, but was found to be a most necessary step in order to prevent the possibility of the genomic DNA fragments acting as templates during the PCR portion of the RT-PCR (Section 3.2.4). Removing the DNA results in fewer false positives and far fewer contaminating background products being produced. This improvement was critical, as the two primers of the Cphsp 2423 & 2764 primer pair are located in the same exon as each other in the *hsp70* gene. This means that if DNA was not removed, genomic DNA and mRNA could not be differentiated, as the same size product would be amplified by the RT-PCR. An RT-negative control (Section 2.5.3.1) was used to help determine whether a false positive had occurred, as no product should be present in this control if contaminating DNA has been fully removed. Persistent genomic DNA was not removed until a specific combination of DNA removal steps, the use of Dynabeads[®] Oligo (dT)₂₅ followed by DNaseI, were introduced (Section 3.2.4). The DNA-free[™] kit (Ambion Inc.) was effective as a DNase Inactivation Reagent was included, which removes the DNaseI and prevents strand scission of the RNA by removing any divalent cations which may be present (Ambion Inc, 2002). The combination of the above steps was the key to the establishment of a truly effective assay as the protocol and reagents in each individual kit were only minimally effective by themselves alone. The wash buffers included with the Dynabeads[®] Oligo (dT)₂₅ (Section 2.3.2) also helped reduce the level of RNase activity. Further, an RNase Inhibitor (Ambion Inc.) was used in the RT-PCR itself to help prevent mRNA degradation (Section 2.5.3.1).

A one-step RT-PCR protocol was chosen because it saves time as the Reverse Transcriptase, *Taq* Polymerase and mRNA were added to the template in a single tube and a master mix was made for the other RT-PCR components. One-step RT-PCR is commonly used for amplifying targets that are reasonably abundant (Ambion Inc, 2004),

which was certainly the case for the *hsp70* gene. As mentioned earlier in this chapter section, the heat shock step increases the expression of *hsp70* mRNA up to a level 10^4 -fold what is normally present in the oocyst (Stinear *et al.*, 1996). The RT-PCR assay was able to detect mRNA from 50 oocysts when 44 cycles of the PCR programme were used (Section 3.2.5.3). Although this does seem a large number of cycles for RT-PCR, no non-specific products were amplified; which often happens when such a high number of cycles are used in conventional PCR protocols. It is possible that using the HotMaster™ *Taq* Polymerase prevented non-specific amplification such as might occur when DNA template and primers are mixed together at a low initial temperature.

When used in the optimised RT-PCR oocyst viability assay protocol the primer pair (Cphsp 2423 & 2764, Table 2.1) provides an assay that is sensitive, reliable and specific for the *C. parvum hsp70* gene (Rochelle *et al.*, 1996). The originally intended application of the assay was for environmental water samples and therefore a low oocyst detection limit was an essential feature of the method. The lowest detection level that was ever achieved was 50 oocysts (Section 3.2.5.4), an unusually large quantity of oocysts for most environmental samples. So unless the RT-PCR assay can be further modified to lower the present oocyst detection limit or a large volume of environmental sample can be concentrated to obtain at least 50 oocysts this application is not possible using the present method. Another reason for wanting to determine the live oocyst detection limit of the assay was so that a log viability reduction could be calculated during UV experiments, as this is how previous studies have reported their results. An aim of this project was to determine a cidal UV dose that could achieve a 3-log reduction in *C. parvum* oocyst viability. This is the level the NZ Water Standards require (Ministry of Health New Zealand, 2000). The observed detection limit of the RT-PCR oocyst viability assay, for oocysts isolated from a petri dish (Section 3.3.2), was between 900 and 5000 oocysts. This means that in order to achieve a 3-log reduction a starting spike of 9×10^5 - 5×10^6 oocysts is required. This is impractical for many reasons; first the cost would be prohibitive over the number of trials required to determine values accurately and, second, shadowing of oocysts from the UV light could occur with such a large number of oocysts in a small volume. As a consequence of the practical restrictions imposed by this detection limit, the present study used 5×10^4 oocysts in each

sample tested during the UV experiments (Section 2.8.2). This quantity could be spiked into petri dishes and was still detected at the end of assay if the sample had not been given a cidal UV dose.

Although the Cphsp 2423 & 2764 primer pair (Table 2.1) was chosen for the level of sensitivity and reliability that it provided the RT-PCR oocyst viability assay, on some occasions two bands (the expected *hsp70* RT-PCR product at 361 bp and another one at ~400 bp) showed on the agarose gels. Rochelle *et al.* (1997) have also noted this phenomenon and commented that it required further investigation. Therefore, although the ~400 bp band was infrequent and appeared not to affect the determination of viability, both of these two bands were sequenced for products obtained for the Camden strain (Section 3.1). One possible reason for the apparent size being ~400 bp in the agarose gel and only a shorter sequence (264 bp) being recovered from sequencing could be the presence of secondary structures. These may have prevented the sequencing reaction and in turn prevented more sequence being determined. Alternatively, undetected errors in the sequencing procedure or data processing may have resulted in the loss of some 3' sequence, as inspection of the sequence shows the 5' end is present but the 3' primer binding site has not been read.

During development trials of the RT-PCR assay (Section 3.2.5.4), the CHSP 1 & 4 primer pair was rejected for use as it was found to be unreliable at amplifying *hsp70* mRNA and at present this cannot be adequately explained. Although Rochelle *et al.* (1997) suggest one possible explanation may lie in the fact that they have an excessive difference (23°C) between the melting temperatures (T_m) of the product and reverse primer, as well as the difference between the T_m of the primers being 12.5°C. These authors found that large differences in primer pair T_m lead to less effective amplification of specific amplicons than primers with closely matched T_m (Rochelle *et al.*, 1997).

One way to satisfy the original objective of developing a quick RT-PCR oocyst viability assay was through the use of a fast gel electrophoresis process. Brody and Kern (2004) noted that both Tris-acetic acid-disodium EDTA (TAE) and Tris boric acid-disodium

EDTA (TBE) buffers are restrictive as electrically conducting media since they generate excessive heat and are of high ionic strength. These factors limit the voltage that can be applied to gels and the speed of electrophoresis (Brody and Kern, 2004). After investigation, the authors found sodium boric acid to be superior to the more conventional agarose gel electrophoresis buffer as it allowed better temperature control and improved convenience by reducing the current drawn from the power supply at any given applied voltage. Although the present study also found good resolution of the RT-PCR product with both TAE and sodium boric acid based buffers (for example, as seen in Figure 3.5) the sodium boric acid buffer was chosen for use in all further experiments as it allowed gel electrophoresis to be completed more quickly (20 minutes compared with 120 minutes) and separated the bands in the 1 Kb Plus DNA ladder well.

4.4 Ultraviolet Light Experiments

Before it could be used in determining a cidal UV dose for *C. parvum*, the improved RT-PCR oocyst viability assay method first needed some further minor modifications (Section 3.3). These changes were necessary because a large sample volume (10 mL) was used in the UV experiments and this needed to be reduced to 100 μ l by using immunomagnetic separation to capture the oocysts from the petri dish followed by a series of washes and concentration steps (Section 2.8) in preparation for the heat shock step of the assay, which was conducted in a PCR tube (Section 2.2.3).

Next, it was important to determine the level of turbidity of the sample solution that oocysts were suspended in to ensure that the actual UV irradiance dose absorbed by oocysts was known as accurately as possible and that relatively little light energy was absorbed by particles of impurities in the solution. Turbidity readings found that DEPC-treated sterile distilled water, with an average reading of 0.05-0.07 NTU, was closest to the accepted level of turbidity by the NZ Water Treatment Standards (Ministry of Health New Zealand, 2000) compared with 1x PBS (with an average reading of 0.9-1.19 NTU). The measurements taken with both the HACH Portable Turbidimeter (Appendix 2) and by Central

Environment Laboratory were in good agreement, showing that the laboratory turbidimeter was properly calibrated and used correctly.

In fact results from a recent study, which applied the RT-PCR oocyst viability assay developed in this study to a detailed investigation of turbidity as an experimental factor, showed how important it was to use a solution with a low turbidity. The aim of the new work was to determine the level of turbidity at which UV was no longer lethal for oocysts. A range of water turbidity levels (0.1-10 NTU) was tested using bentonite added to the DEPC-treated water used to suspend oocysts in the petri dish during UV exposure. A lethal UV dose was used to irradiate 5×10^4 viable oocysts and preliminary results confirm that as turbidity increases, the number of oocysts killed decreases (Pita, 2005).

Consistent delivery of UV dose during exposure of oocysts was an important consideration for accurate lethal dose determination and absolutely necessary in order to obtain comparable results between trials. The output of any UV lamp varies differently over both the short and long term (United States Environmental Protection Agency *et al.*, 2003). There is a natural diminution of UV irradiance emitted from a UV lamp over time (lamp aging), however, even after approximately 10 000 hours of use, a lamp should still emit around half of its initial output level (United States Environmental Protection Agency *et al.*, 2003). Therefore, lamp aging was not considered to be a significant issue for the experimental design of this part of the study. Continual readings could not be taken during UV exposure, so readings immediately before and after irradiation were obtained. As expected, there was an increase (approximately 4%) in the reading taken post-irradiance, which could be attributed to the lamp having heated up during the exposure time.

A lethal UV dose was judged to have been achieved when an RT-PCR product band was observed for the positive non-irradiated oocyst control and when no band was present for the UV-irradiated oocyst sample, see for example Figure 3.6. The experiment (Section 2.8) used a range of UV doses, starting from 10 mJ/cm^2 because this is close to that which previous studies have found to achieve a 3-log reduction in *C. parvum* infectivity when animal infectivity and cell culture viability assay methods were employed (Clancy *et al.*,

2000; Shin *et al.*, 2001). Results from the set of experiments reported here (Table 3.5) using the RT-PCR oocyst viability assay, do not corroborate results from these previous studies. Doses as high as 1100 mJ/cm² were required to be reliably cidal for oocysts.

On close inspection of the gel in Figure 3.6, Lanes 6 & 7, a dose of 750 mJ/cm² showed one positive and one negative result, which may indicate that this dose was close to the cidal dose for oocysts. At the time when these results were obtained, the primary aim of the tests was to determine a cidal dose for oocysts so that investigations of the photoreactivation process could be started. Therefore, doses between 750 mJ/cm² and 1100 mJ/cm² were not tested, but it would be of value to investigate this range of doses in future experiments in order to obtain a more accurate cidal dose for Camden oocysts.

No comment can be made regarding the relative UV susceptibility of the two strains (Camden and Iowa), as only a preliminary trial was conducted to determine a cidal dose for the Iowa strain (Section 3.3.4). However, Clancy *et al.* (2004) report that strains with the same genotype do have similar UV susceptibility. Thus it might be expected that Iowa oocysts would have had a similar cidal dose to Camden oocysts under the conditions used in this experiment since they are both of the bovine genotype (Section 3.1).

A limited trial was performed using the β -tubulin gene as an RT-PCR target to see if an alternative genetic marker of viability could also be used when studying UV inactivation of oocysts. The β -tubulin gene was chosen because its mRNA is not produced in response to stress as with *hsp70* mRNA, which could reduce the apparent cidal UV dose required for oocysts. The results obtained during the β -tubulin trials showed that a UV dose as low as 10 mJ/cm² could be cidal for oocysts. This observation is particularly interesting since this dose is consistent with data obtained in research conducted using mouse infectivity to determine oocyst viability (Bukhari *et al.*, 1999; Clancy *et al.*, 2000). This cidal UV dose (10 mJ/cm²) is much lower than the result obtained when the *hsp70* gene is used (1100 mJ/cm²) in the RT-PCR assay (Section 3.3.4). An explanation for this could be that there was such a high level of *hsp70* mRNA present after heat shock that when expression of this gene was used as a viability marker, it appeared that a much higher dose of UV might be

required to be cidal for oocysts (Section 3.3.4). This could be caused by a difference in the post mortem half-life of the two mRNA species. Perhaps β -tubulin mRNA has a shorter half-life than *hsp70* mRNA within the oocyst and therefore even when a low UV dose is given, β -tubulin mRNA is only present at a level which is below the detection threshold of the RT-PCR assay, producing a negative result.

When examining the results from the β -tubulin gene trials (shown in Figure 3.8), the inferred presence of β -tubulin mRNA seen at a dose of 30 mJ/cm² (Lane 4) could be explained as a false positive. It is unlikely that the 40 mJ/cm² result (Lane 5) was a false negative since several other trials were previously conducted at 40, 200 and 400 mJ/cm² and were all negative, indicating these doses were cidal for oocysts. In designing future experiments, it would be desirable to include more replicates to reduce the potential impact of false results.

Once the cidal UV dose had been determined (Section 3.3.4), the possibility of photoreactivation could be investigated. A wavelength of 365 nm is widely regarded as the wavelength at which photorepair of DNA is stimulated which if extensive enough can lead to photoreactivation of *Cryptosporidium* (Clever, 2003; Kashimada *et al.*, 1996; Lindenauer and Darby, 1994; Oguma *et al.*, 2001; Oguma, 2002; Shin *et al.*, 2001). Essential preliminary tests were performed to ensure that exposure to 365 nm light was not detrimental to *C. parvum* oocysts as it was not clear whether the above mentioned studies had tested this. Photoreactivation did not occur in the small number of samples processed during this study but maybe with more replicates evidence of photoreactivation could have been obtained. The present results corroborate well with those obtained in several other studies (Oguma *et al.*, 2001; Rochelle *et al.*, 2005; Shin *et al.*, 2001) that found no photoreactivation in *C. parvum*. In contrast, Oguma *et al.* (2002) used an assay that quantified the amount of genetic damage and found evidence indicating that photorepair can occur. Another idea to consider is that the very high cidal UV dose (1100 mJ/cm²) employed here may have prevented photoreactivation from occurring because the damage to the oocyst was too extensive for the oocyst DNA repair systems to be able to regain full metabolic activity. Thus slightly lower UV doses, which still showed as being cidal or

close to cidal using the RT-PCR oocyst viability assay, may create less genetic damage and could allow for photorepair.

4.4.1 The Difference Between Inactivation and Metabolic Activity

The oocyst is metabolically dormant but is capable of becoming metabolically active although not always infective (Jenkins *et al.*, 1997). Therefore, the definition of oocyst inactivation itself needs to be investigated in depth, as there is clearly a difference between an oocyst being **metabolically active, but not infective** and **metabolically inactive altogether**. The difference seen between the cidal UV dose data obtained for this project and those that are routinely published could be due to the use of these different inactivation definitions. Does a band on an agarose gel show the inferred presence of mRNA because the oocyst is still metabolically active or because the mRNA is stable within the oocyst and has not degraded? Maybe the other studies reported much lower cidal doses because they have made the oocysts non-infective whereas the cidal dose determined in this project was rendering the oocysts metabolism completely inactive.

Smith *et al.* (2004) discussed the idea that the choice of inactivation method used on *C. parvum* oocysts would determine which viability assay should be used. For example, the fluorescent *in situ* hybridisation assay used by Smith *et al.* (2004) shows false positives when UV is used to inactivate oocysts. They suggested that perhaps this observation should then require the use of more than one viability assay so that there can be corroboration of results (Smith *et al.*, 2004).

More specifically, the United States Environmental Protection Agency (2003) stated that they believed it was important to use assays that measure infectivity and not viability when studying the effectiveness of UV disinfection. They cited the past cases of UV disinfection being viewed as ineffective for inactivating *Cryptosporidium* because early researchers used assays that tested only viability (excystation and vital dyes) and not infectivity. In fact very low doses of UV light (20 mJ/cm²), giving a 4-log inactivation of *Cryptosporidium*, were shown to be effective by Bukhari *et al.* (1999) when infectivity assays were used.

4.5 Heat Shock and Heat Inactivation Conditions

In this study inclusion and exclusion of the heat shock step (12 minutes at 45°C) (Section 2.2.3) prior to oocyst lysis produced no visual difference in the amount of RT-PCR product obtained from mRNA present. This result is the same as that found by Stinear *et al.* (1996). But later authors (Rochelle *et al.*, 1997) suggested that these earlier results could be attributed to constitutive expression of the *hsp70* gene in *C. parvum* because the 5' non-coding part of the gene does not contain the ideal regulatory heat shock element common to most *hsp70* genes.

The RT-PCR oocyst viability assay is semi-quantitative because it uses visual comparison of RT-PCR product band intensities on an agarose gel. Therefore, it is quite possible that there is a high level of mRNA in a sample of 5×10^4 oocysts and this may be the reason no difference is seen between tests with inclusion or exclusion of the heat shock step in the assay. This idea was investigated in Section 3.4 by exposing various quantities of oocysts (1000 - 5×10^4) to a heat shock step (12 minutes at 45°C) or not doing so, prior to continuing with the RT-PCR oocyst viability assay (Sections 2.2.4.2, 2.3.2, 2.6.1, 2.5.3.1). As oocyst numbers between 3000 and 1000 were not tested, more conclusive results could not be obtained on the effect of the number of oocysts and their effect in producing visual differences in results between heat shock and no heat shock procedures. Future work should be conducted using this range of oocyst numbers since this may well show a difference. Using less than 1000 oocysts would seem to be of no value as this is below the detection limit of the RT-PCR oocyst viability assay when oocysts are isolated from a petri dish (Table 3.3). Future use of real-time RT-PCR could be investigated to see if there are real differences in the amount of *hsp70* mRNA present with and without the heat shock step. Perhaps a difference could also be seen if the number of RT-PCR cycles was reduced from 44 cycles (Section 2.5.3.1), as this may lack sensitivity since the reaction would be expected to have reached the stationary phase. Real-time RT-PCR would return results for the exponential phase.

The use of heat for inactivation of *C. parvum* was investigated by exposing oocysts to 20 minutes at 100°C followed by the standard RT-PCR oocyst viability assay (Sections 2.2.4.2, 2.3.2, 2.6.1, 2.5.3.1). Unlike the results found by Hallier-Soulier and Guillot (2003) and Kaucner and Stinear (1998), *hsp70* mRNA was still detectable after heat inactivation in the present study. Another study (Mahbubani *et al.*, 1991) has also reported that mRNA was still detectable after *Giardia* cysts were heat treated, although this was in an investigation of giardin protein expression in *Giardia*. Quite different results were obtained when the β -tubulin gene was used in the present study. After heat inactivation no β -tubulin mRNA could be detected (Figure 3.10, Lane 4). This implies that the method of oocyst inactivation could be a factor, which might explain why there is a difference seen in the level of expression of these two genes. Heating to 100°C would stress oocysts and would in turn raise levels of *hsp70* mRNA. As the time taken to reach this temperature was only 50 seconds (since these experiments were performed in a thermal cycler) enzymes ordinarily responsible for degradation of mRNA within oocysts would be inactivated before the majority of *hsp70* mRNA could be removed. One way to test this theory could be to reduce the ramp speed of the thermal cycler to allow the cellular enzymes time to remove the *hsp70* mRNA. If the preceding ideas are correct then *hsp70* mRNA may not be detected when the RT-PCR oocyst viability assay is used, as the enzymes will have time to remove the mRNA and the level present may be under the detection limit of the RT-PCR.

Therefore, use of a different method to inactivate oocysts (e.g. UV, formalin, chlorine) followed by the RT-PCR assay using the *hsp70* gene as a viability marker could provide a more effective means to distinguish differences between non-viable and viable oocysts by avoiding the effect that heat has on *hsp70* mRNA production. For example, Stinear *et al.* (1996) used an RT-PCR assay and found that formalin-treated oocysts contained no detectable *hsp70* mRNA. Chlorination, chlorine dioxide and ozonation treatments for inactivation of oocysts have all been tested previously by Hallier-Soulier and Guillot (2003) who also used RT-PCR detection of *hsp70* mRNA to test oocyst viability. They found the RT-PCR signal disappeared with ozonation, but did not disappear with chlorination or chlorine dioxide. However, the contact times used for both of these last two reagents by the above workers were recognised as not being sufficient for inactivation by another study

(Korich *et al.*, 1990). Therefore, since it is unknown to what extent chemical treatments stress oocysts, conducting an empirical study using RT-PCR with the *hsp70* gene as a marker would be beneficial.

The stability of *hsp70* mRNA within inactivated oocysts is an important consideration in assay design especially with respect to potential false positive results. For example, an oocyst sample might be deemed viable because mRNA is detected when in fact a stable mRNA simply persists inside a non-viable oocyst. However, as stated earlier a false positive would always be preferable to a false negative result in a public health situation. This study found that even after 70 hours at room temperature *hsp70* mRNA was still present in some heat inactivated oocysts (Figure 3.11, Lane 2). This finding is in contrast to several other studies (Baeumner *et al.*, 2001; Hallier-Soulier and Guillot, 2003; Stinear *et al.*, 1996) that have reported that dead *C. parvum* oocysts do not contain detectable *hsp70* mRNA. Their conclusions could have been reached because their studies used methods such as formalin or ozone rather than heat inactivation to kill the oocysts. Interestingly, Baeumner *et al.* (2001) found that *hsp70* mRNA was detected up to 30 minutes after what they termed, “heat killing” (15 minutes at 95°C). Indeed, one sample was still detected as positive at 3 hours post treatment but mRNA could not be detected from any samples after 24 hours. Fontaine and Guillot (2003) hypothesised that even with a 95°C heat treatment cellular structures such as the oocyst wall and the ribosomal subunits would remain intact. The latter are protected by the combined effect of the nucleic acid secondary structure and ribosomal proteins. Similar consideration might explain the extended stability of *hsp70* mRNA (70 hours at room temperature) found in the present study, as mRNA degradation due to internal and external sources would be prevented.

Therefore, when testing the viability of oocysts using heat inactivation targeting the β -tubulin gene with the RT-PCR oocyst viability assay provides better accuracy. The *hsp70* gene with the RT-PCR is more appropriate to use when oocysts are inactivated with a method other than heat such as UV (Section 3.3.4 and Figure 3.6). The stability of *hsp70* mRNA within oocysts could be more accurately investigated by using UV to inactivate oocysts instead of heat inactivation as in the present study.

The RT-PCR oocyst viability assay developed in this study has been shown to work effectively. The choice of the target gene to be used in the assay has been refined and the discovery made that both the *hsp70* and β -tubulin genes could potentially be used depending on the method of oocyst inactivation that was used. The cidal dose for *C. parvum* oocysts was determined with some interesting differences found with respect to previous work conducted. The overall success of the work conducted in this project will ultimately open up other future research prospects, e.g. such as the already conducted experiments using different levels of turbidity (see earlier in Section 4.4) or using different oocyst inactivation methods in conjunction with the RT-PCR oocyst viability assay and comparing these results to those obtained with other viability testing methods. Another future objective could be to develop this assay into a high throughput method for use as a routine diagnostic screening test for environmental water samples. Additionally, the availability of the complete *C. parvum* genome sequence now makes it possible for researchers to search for other candidate loci to be used as viability markers.

APPENDICES

Appendix 1: Common Buffers and Solutions

Unless otherwise stated all buffers and solutions were prepared using RO water. The pH was adjusted as indicated and the solutions were stored at room temperature.

1.1 1x TAE buffer (pH 7.8)

39 mM Tris-HCl, 4.9 mM Na₂EDTA and 1 mM anhydrous sodium acetate. The pH was adjusted to pH 7.8 with glacial acetic acid.

1.2 1x sodium boric acid (pH 8.5)

10 mM NaOH and the pH was adjusted to 8.5 with boric acid (Brody and Kern, 2004).

1.3 1x TE buffer

10 mM Tris-HCl and 1 mM Na₂EDTA.

1.4 TE buffer and 1% (v/v) Nonidet P40

1% (v/v) Nonidet P40 (Roche Diagnostics, Germany) in TE Buffer.

1.5 Phosphate buffered saline (PBS) (pH 7.2)

145 mM NaCl, 7.53 mM Na₂HPO₄.12H₂O, 2.48 mM NaH₂HPO₄.2H₂O.

1.6 20% (w/v) Chelex[®]

20% (w/v) Chelex[®] (BioRad, USA).

1.7 10x gel loading dye

20% (w/v) glycerol, 1 mM EDTA (pH 8.0) and 0.25% bromophenol blue.

Appendix 2: Turbidity Readings of DEPC-Treated Water and 1x PBS Buffer

Turbidity readings of the water (Section 2.4.2) and 1x PBS buffer (Appendix 1.5) (both treated with 0.01% DEPC), used to determine the cidal UV dose for *C. parvum* oocysts, were measured using a HACH Portable Turbidimeter (HACH Company, USA) (Appendix 3.3) following the manufacturers protocol. The sample was degassed prior to a reading being taken.

Serial dilutions of bentonite (Sigma-Aldrich Pty. Ltd, Sydney, Australia) taken from a 1% (w/v) solution were also analysed by Central Environment Laboratory (CEL) Palmerston North, an International Accreditation New Zealand (IANZ) accredited laboratory, where the turbidity readings were referenced against a certificated turbidimeter.

Appendix 3: Equipment Details

3.1 Magnetic tube holders (Institute of Fundamental Sciences Engineering Services Workshop, Massey University, Palmerston North, NZ)

- Small; holds a microcentrifuge tube
- Large; holds a 15 mL tube

3.2 Spectroline[®] E-Series Ultraviolet Hand Lamps (Spectroline[®])

- Model X-15G/F (254nm, peak intensity at 25 cm is 820 $\mu\text{W}/\text{cm}^2$, 240 Volts, 15 Watt, 50 Hz, 0.40 Amps. Dimensions: 11.4cm x 47.6cm x 8.9 cm, 2kg)
- Model EA-180/FE (365 nm, peak intensity at 15 cm is 1700 $\mu\text{W}/\text{cm}^2$, 230 Volts, 8 Watt, 50 Hz, 0.17 Amps)

3.3 Turbidimeter (HACH Company, USA)

- Model 2100P

3.4 UV detectors (International Light, Newburyport, USA)

- 254 nm (Model SEL240/QNDS2/TD, with a spectral range of 185-310 nm)
- 365 nm (SEL033/UVA/W, with a spectral range of 315-390 nm)

Appendix 4: Equations

4.1 Enumeration of *C. parvum* Oocysts per mL of Suspension

Taken from USEPA Method 1622, version 2001 (United States Environmental Protection Agency, 2001).

$\frac{\text{Number of organisms counted}}{\text{Number of mm}^2 \text{ counted}} \times \frac{10}{1 \text{ mm}} \times \frac{\text{Dilution factor}}{1} \times \frac{1000 \text{ mm}^3}{1 \text{ mL}}$
<p>= Number of organisms/mL</p>

4.2 Determination of UV Dose

Adapted from (United States Environmental Protection Agency *et al.*, 2003).

$$\text{UV Dose (mJ/cm}^2\text{)} = I \times t$$

Where:

I = UV intensity (mW/cm²)

t = Exposure time (s)

[NB. 1mWs/cm² = 1 mJ/cm²]

REFERENCES

- Abbaszadegan, M., Huber, M., Gerba, C., and Pepper, I.** (1997) Detection of Viable *Giardia* Cysts by Amplification of Heat Shock-Induced mRNA. *Applied and Environmental Microbiology* **63**: 324-328.
- Ambion Inc** (2002) DNA-free product manual., 1-12.
- Ambion Inc** (2004) Cells-to-cDNA II product manual., 1-27.
- Baumner, A.J., Humiston, M.C., Montagna, R.A., and Durst, R.A.** (2001) Detection of Viable Oocysts of *Cryptosporidium parvum* Following Nucleic Acid Sequence Based Amplification. *Analytical Chemistry* **73**: 1176-1180.
- Bolton, J.** (2000). Terms and Definitions in Ultraviolet Disinfection. Disinfection 2000. New Orleans
- Bolton, J.** (2004) UV FAQs. *IUVA News* **6**: 35-36.
- Bolton, J., and Linden, K.** (2002) Standardization of Methods for Fluence (UV Dose) Determination in Bench-Scale UV Experiments. *Journal of Environmental Engineering* **In Press**.
- Brody, J., and Kern, S.** (2004) Sodium Boric Acid: A Tris-free, Cooler Conductive Medium for DNA Electrophoresis. *BioTechniques* **36**: 214-216.
- Bukhari, Z., Hargy, T., Bolton, J., Dussert, B., and Clancy, J.** (1999) Medium-Pressure UV for Oocyst Inactivation. *Journal of American Water Works Association* **91**: 86-94.

Bukhari, Z., and LeChevallier, M. (2004) Finished Water Disinfection with UV Light: Overview of Validation Studies at American Water. *IUVA News* **6**: 15-20.

Caccio, S., Homan, W., van Dijk, K., and Pozio, E. (1999) Genetic Polymorphism at the β -tubulin Locus Among Human and Animal Isolates of *Cryptosporidium parvum*. *FEMS Microbiology Letters* **170**: 173-179.

Call, J., Arrowood, M., Long-Ti, X., Hancock, K., and Tsang, V. (2001) Immunoassay for Viable *Cryptosporidium parvum* Oocysts in Turbid Environmental Water Samples. *The Journal of Parasitology* **87**: 203-210.

Campbell, A., Robertson, L., and Smith, H. (1992) Viability of *Cryptosporidium parvum* Oocysts: Correlation of *in vitro* Excystation with Inclusion or Exclusion of Vital Dye. *Applied and Environmental Microbiology* **58**: 3488-3493.

Carey, C., Lee, H., and Trevors, J. (2004) Biology, Persistence and Detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* Oocyst. *Water Research* **38**: 818-862.

Center for Disease Control and Prevention CDC Parasitology Diagnostic Web Site.
URL <http://www.dpd.cdc.gov/dpdx/HTML/Cryptosporidiosis.asp>

Chambers, C.D. (2002) Unpublished Data.

Chung, E., Aldom, J., Carreno, R., Chagla, A., Kostrzynska, M., Lee, H. et al. (1999) PCR - Based Quantitation of *Cryptosporidium parvum* in Municipal Water Samples. *Journal of Microbiological Methods* **38**: 119 - 130.

Cinque, K., Stevens, M., Roser, D., Ashbolt, N., and Leeming, R. (2004) Assessing the Health Implications of Turbidity and Suspended Particles in Protected Catchments. *Water Science and Technology* **50**: 205-210.

Clancy, J. (1998). Comparison of Viability and Infectivity Methods for *Cryptosporidium* in the U.S. and U.K. AWWA Water Quality Technology Conference. San Diego, California

Clancy, J., Bukhari, Z., Hargy, T., Bolton, J., Dussert, B., and Marshall, M. (2000) Using UV to Inactivate *Cryptosporidium*. *Journal of American Water Works Association* **92**: 97-104.

Clancy, J., Marshall, M., Hargy, T., and Korich, D. (2004) Susceptibility of Five Strains of *Cryptosporidium parvum* Oocysts to UV Light. *Journal of American Water Works Association* **96**: 84-93.

Cleaver, J. (2003) Classics in DNA Repair: Photoreactivation. *DNA Repair* **2**: 629-638.

Cotton, C., Owen, D., Cline, G., and Brodeur, T. (2001) UV Disinfection Costs for Inactivating *Cryptosporidium*. *Journal of American Water Works Association* **93**: 82-94.

Craik, S., Weldon, D., Finch, G., Bolton, J., and Belosevic, M. (2001) Inactivation of *Cryptosporidium Parvum* Oocysts Using Medium- and Low-Pressure Ultraviolet Radiation. *Water Research* **35**: 1387-1398.

Dawson, D., Samuel, C., Scrannage, V., and Atherton, C. (2004) Survival of *Cryptosporidium* Species in Environments Relevant to Foods and Beverages. *Journal of Applied Microbiology* **96**: 1222-1229.

de Graaf, D., Spano, F., Petry, F., Sagodira, S., Bonnin, A., and Crisanti, A. (1999) Speculation on Whether a Vaccine Against Cryptosporidiosis is a Reality or a Fantasy. *International Journal for Parasitology* **29**: 1289 - 1306.

Downes, A., and Blunt, T.P. (1877) Researches on the Effect of Light Upon Bacteria and Other Organisms. *Proceedings of the Royal Society of London* **26**: 488-500.

Drescher, A., Greene, D., and Gadgil, A. (2001) *Cryptosporidium* Inactivation by Low-Pressure UV in a Water Disinfection Device. *Journal of Environmental Health* **64**: 31-35.

Dynal Biotech (2002) Dynabeads GC-Combo Package Insert. Oslo, Norway.

Fayer, R. (1994) Effect of High Temperature on Infectivity of *Cryptosporidium parvum* Oocysts in Water. *Applied and Environmental Microbiology* **60**: 2732 - 2735.

Fayer, R., Morgan, U., and Upton, S. (2000) Epidemiology of *Cryptosporidium*: Transmission, Detection and Identification. *International Journal for Parasitology* **30**: 1305-1322.

Fayer, R., and Nerad, T. (1996) Effects of Low Temperatures on Viability of *Cryptosporidium parvum* Oocysts. *Applied and Environmental Microbiology* **62**: 1431-1433.

Fayer, R., Trout, J., and Nerad, T. (1996) Effects of a Wide Range of Temperatures on Infectivity of *Cryptosporidium parvum* Oocysts. *Journal of Eukaryotic Microbiology* **43**: 64S.

Fontaine, M., and Guillot, E. (2003) Study of 18S rRNA and rDNA Stability by Real-Time RT-PCR in Heat-Inactivated *Cryptosporidium parvum* Oocysts. *FEMS Microbiology Letters* **226**: 237-243.

Gobet, P., and Toze, S. (2001) Relevance of *Cryptosporidium parvum* *hsp70* mRNA Amplification as a Tool to Discriminate Between Viable and Dead Oocysts. *The Journal of Parasitology* **87**: 226-229.

Goin, J., Skirda, A., and Tohktuev, E. (2004) Understanding UV Monitoring for Air and Water UV Treatments. *IUVA News* **6**: 29-35.

Hallier-Soulier, S., and Guillot, E. (2003) An Immunomagnetic Separation-Reverse Transcription Polymerase Chain Reaction (IMS-RT-PCR) Test for Sensitive and Rapid Detection of Viable Waterborne *Cryptosporidium parvum*. *Environmental Microbiology* **5**: 592-598.

Hargy, T., Clancy, J., Bukhari, Z., and Marshall, M. (2000) Shedding UV Light on the *Cryptosporidium* Threat. *Journal of Environmental Health* **63**: 19-22.

Harp, J., Fayer, R., Pesch, B., and Jackson, G. (1996) Effect of Pasteurisation on Infectivity of *Cryptosporidium parvum* Oocysts in Water and Milk. *Applied and Environmental Microbiology* **62**: 2866 - 2868.

International Light (1998) IL1400A Radiometer Instruction Manual. Newburyport, MA, USA.

Ionas, G. (2002) Personal Communication.

Jenkins, M., Anguish, L., Bowman, D., Walker, M., and Ghiorse, W. (1997) Assessment of Dye Permeability Assay for Determination of Inactivation Rates of *Cryptosporidium parvum* Oocysts. *Applied and Environmental Microbiology* **63**: 3844-3850.

Jenkins, M., Trout, J., Higgins, J., Dorsch, M., Veal, D., and Fayer, R. (2003) Comparison of Tests for Viable and Infectious *Cryptosporidium parvum* Oocysts. *Parasitology Research* **89**: 1-5.

Karanis, P., Maier, W., Seitz, H., and Schoenen, D. (1992) UV Sensitivity of Protozoan Parasites. *Aqua* **41**: 95-100.

Kashimada, K., Kamiko, N., Yamamoto, K., and Ohgaki, S. (1996) Assessment of Photoreactivation Following Ultraviolet Light Disinfection. *Water Science and Technology* **33**: 261-269.

Kaucner, C., and Stinear, T. (1998) Sensitive and Rapid Detection of Viable *Giardia* Cysts and *Cryptosporidium parvum* Oocysts in Large-Volume Water Samples with Wound Fiberglass Cartridge Filters and Reverse Transcription-PCR. *Applied and Environmental Microbiology* **64**: 1743-1749.

Kelner, A. (1949) Effect of Visible Light on the Recovery of *Streptomyces griseus* Conidia from Ultraviolet Irradiation Injury. *Proceedings of the National Academy of Sciences* **35**: 73-79.

Kim, H., and Healey, M. (2001) Infectivity of *Cryptosporidium parvum* Oocysts Following Cryopreservation. *Journal of Parasitology* **87**: 1191-1194.

Kim, J., and Sundin, G. (2001) Construction and Analysis of Photolyase Mutants of *Pseudomonas aeruginosa* and *Pseudomonas syringae*: Contribution of Photoreactivation, Nucleotide Excision Repair, and Mutagenic DNA Repair to Cell Survival and Mutability Following Exposure to UV-B Radiation. *Applied and Environmental Microbiology* **67**: 1405-1411.

Korich, D., Mead, J., Madore, M., Sinclair, N., and Sterling, C. (1990) Effects of Ozone, Chloride Dioxide, Chlorine and Monochloramine on *Cryptosporidium parvum* Oocyst Viability. *Applied and Environmental Microbiology* **56**: 1423-1428.

Linden, K., Shin, G., and Sobsey, M. (2001) Comparative Effectiveness of UV Wavelengths for the Inactivation of *Cryptosporidium parvum* Oocysts in Water. *Water Science and Technology* **43**: 171-174.

Lindenauer, K., and Darby, J. (1994) Ultraviolet Disinfection of Wastewater: Effect of Dose on Subsequent Photoreactivation. *Water Research* **28**: 805-817.

Lorenzo-Lorenzo, M., Ares-Mazas, M., Villacorta-Martinez de Maturana, I., and Duran-Oreiro, D. (1993) Effect of Ultraviolet Disinfection of Drinking Water on the Viability of *Cryptosporidium parvum* Oocysts. *The Journal of Parasitology* **79**: 67-70.

Mackey, E., Hargy, T., Wright, H., Malley, J., and Cushing, R. (2002) Comparing *Cryptosporidium* and MS2 Bioassays - Implications for UV Reactor Validation. *Journal of American Water Works Association* **94**: 62-69.

Mackey, E., Malley, J., Hargy, T., and Cushing, R. (2000). MS-2 Bioassays and *Cryptosporidium* Challenges: Comparing and Contrasting UV Reactor Validation Techniques. American Water Works Association Water Quality Conference Proceedings.

Mahbubani, M.H., Bej, A.K., Perlin, M., Schaefer III, F.W., Jakubowski, W., and Atlas, R.M. (1991) Detection of *Giardia* Cysts by Using the Polymerase Chain Reaction and Distinguishing Live from Dead Cysts. *Applied and Environmental Microbiology* **57**: 3456-3461.

Martins, C.A.P., and Guerrant, R.L. (1995) *Cryptosporidium* and Cryptosporidiosis. *Parasitology Today* **11**: 434 - 436.

Mead, J., Bonafonte, M., Arrowood, M., and Schinazi, R. (1996) *In vitro* Expression of mRNA Coding for a *Cryptosporidium parvum* Oocyst Wall Protein. *Journal of Eukaryotic Microbiology* **43**: 84S.

Meisel, J.L., Perera, D.R., Meligro, C., and Robin, C.E. (1976) Overwhelming Diarrhoea Associated with a *Cryptosporidium* in an Immunosuppressed Patient. *Gastroenterology* **70**: 1156-1160.

Ministry of Health New Zealand (2000) Drinking-Water Standards For New Zealand 2000. Wellington, New Zealand: Ministry of Health.

Mofidi, A., Baribeau, H., Rochelle, P., De Leon, R., Coffey, B., and Green, J. (2001) Disinfection of *Cryptosporidium parvum* with Polychromatic UV Light. *Journal of American Water Works Association* **93**: 95-109.

Morgan, U., and Thompson, R. (1998) PCR Detection of *Cryptosporidium*: The Way Forward? *Parasitology Today* **14**: 241-245.

National Institute of Standards and Technology (2004). NIST Policy on Traceability. URL <http://ts.nist.gov/traceability/>

Nime, F.A., Burek, J.D., Page, D.L., Holscher, M.A., and Yardley, J.H. (1976) Acute Enterocolitis in a Human Being Infected with the Protozoan *Cryptosporidium*. *Gastroenterology* **70**: 592-598.

Nwachuku, N., and Gerba, C. (2004) Emerging Waterborne Pathogens: Can We Kill Them All? *Current Opinion in Biotechnology* **15**: 175-180.

Oguma, K., Katayama, H., Mitani, H., Morita, S., Hirata, T., and Ohgaki, S. (2001) Determination of Pyrimidine Dimers in *Escherichia coli* and *Cryptosporidium parvum* During UV Light Inactivation, Photoreactivation and Dark Repair. *Applied and Environmental Microbiology* **67**: 4630-4637.

Oguma, K., Katayama, H., Ohgaki, S. (2002) Photoreactivation of *Escherichia coli* After Low- or Medium-Pressure UV Disinfection Determined by an Endonuclease Sensitive Site Assay. *Applied and Environmental Microbiology* **68**: 6029-6035.

Okhuysen, P., Chappell, C., Crabb, J.H., Sterling, C., and Dupont, H.L. (1999) Virulence of Three Distinct *Cryptosporidium parvum* Isolates for Healthy Adults. *The Journal of Infectious Diseases* **180**: 1275-1281.

Pita, A. (2005) Personal Communication.

Quintero-Betancourt, W., Peele, E., and Rose, J. (2002) *Cryptosporidium parvum* and *Cyclospora cayentanensis*: A Review of Laboratory Methods for Detection of These Waterborne Parasites. *Journal of Microbiological Methods* **49**: 209-224.

Robertson, L., Campbell, A., and Smith, H. (1992) Survival of *Cryptosporidium parvum* Oocysts Under Various Environmental Pressures. *Applied and Environmental Microbiology* **58**: 3494 - 3500.

Robertson, L., and Gjerde, B. (2004) Effects of the Norwegian Winter Environment on *Giardia* Cysts and *Cryptosporidium* Oocysts. *Microbial Ecology* **47**: 359-365.

Rochelle, P., De Leon, R., Johnson, A., Stewart, M., and Wolfe, R. (1999) Evaluation of Immunomagnetic Separation for Recovery of Infectious *Cryptosporidium parvum* Oocysts from Environmental Samples. *Applied and Environmental Microbiology* **65**: 841-845.

Rochelle, P., De Leon, R., Stewart, M., and Wolfe, R. (1997) Comparison of Primers and Optimization of PCR Conditions for Detection of *Cryptosporidium parvum* and *Giardia lamblia* in Water. *Applied and Environmental Microbiology* **63**: 106-114.

Rochelle, P., Ferguson, D., Handojo, T., De Leon, R., Stewart, M., and Wolfe, R. (1996) Development of a Rapid Detection Procedure for *Cryptosporidium*, Using *in vitro* Cell Culture Combined with PCR. *Journal of Eukaryotic Microbiology* **43**: 72S.

Rochelle, P., Ferguson, D., Handojo, T., De Leon, R., Stewart, M., and Wolfe, R. (1997) An Assay Combining Cell Culture with Reverse Transcriptase PCR to Detect and Determine the Infectivity of Waterborne *Cryptosporidium parvum*. *Applied and Environmental Microbiology* **63**: 2029-2037.

Rochelle, P., Marshall, M., Mead, J., Johnson, A., Korich, D., Rosen, J., and De Leon, R. (2002) Comparison of *in vitro* Cell Culture and a Mouse Assay for Measuring Infectivity of *Cryptosporidium parvum*. *Applied and Environmental Microbiology* **68**: 3809-3817.

Rochelle, P., Upton, S., Montelone, B.A., and Woods, K.M. (2005) The Response of *Cryptosporidium parvum* to UV Light. *Trends in Parasitology* **21**: 81-87.

Rose, J., Huffman, D., and Gennaccaro, A. (2002) Risk and Control of Waterborne Cryptosporidiosis. *FEMS Microbiology Review* **26**: 113-123.

Shaw, M., and Warne, S. (2003). A Comparison of Microbial Repair Mechanisms with Low Pressure and Medium Pressure UV Lamps. *Water & Wastes In New Zealand*. November. 50-52.

Shin, G., Linden, K., Arrowood, M., and Sobsey, M. (2001) Low-Pressure UV Inactivation and DNA Repair Potential of *Cryptosporidium parvum* Oocysts. *Applied and Environmental Microbiology* **67**: 3029-3032.

Slifko, T., Friedman, D., Rose, J., and Jakubowski, W. (1997) An *in vitro* Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture. *Applied and Environmental Microbiology* **63**: 3669-3675.

Smith, J.J., Gunasekera, T., Barardi, C., Veal, D., and Vesey, G. (2004) Determination of *Cryptosporidium parvum* Oocyst Viability by Fluorescence *in situ* Hybridisation Using a Ribosomal RNA-Directed Probe. *Journal of Applied Microbiology* **96**: 409-417.

Stinear, T., Matusan, A., Hines, K., and Sandery, M. (1996) Detection of a Single Viable *Cryptosporidium parvum* Oocyst in Environmental Water Concentrates by Reverse Transcription-PCR. *Applied and Environmental Microbiology* **62**: 3385-3390.

Taylor, M. (2003). UV Disinfection of Drinking Water (Draft). Ministry of Health New Zealand. Wellington. 1-14

Tyzzar, E.E. (1907) A Sporozoan Found in the Peptic Glands of the Common Mouse. *Proceedings of the Society for Experimental Biology and Medicine*. **5**: 12-13.

United States Environmental Protection Agency. (2001a). Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA. United States Environmental Protection Agency. 1-51

United States Environmental Protection Agency. (2001b). Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. United States Environmental Protection Agency. Washington DC. 1-52

United States Environmental Protection Agency, Linden, K., Malley, J., Malcolm Pirnie Inc, Carollo Engineers, and The Cadmus Group Inc. (2003). Ultraviolet Disinfection Guidance Manual: June 2003 Draft. United States Environmental Protection Agency. Washington DC. 1-478

Veal, D., Deere, D., Ferrari, B., Piper, J., and Attfield, P. (2000) Fluorescence Staining and Flow Cytometry for Monitoring Microbial Cells. *Journal of Immunological Methods* **243**: 191 - 210.

Vesey, G., Ashbolt, N., Fricker, C., Deere, D., Williams, K., Veale, D., and Doesch, M. (1998) The Use of a Ribosomal RNA Targeted Oligonucleotide Probe for Fluorescent Labelling of Viable *Cryptosporidium parvum* Oocysts. *Journal of Applied Bacteriology* **85**: 429-440.

Wagner-Wiening, C., and Kimmig, P. (1995) Detection of Viable *Cryptosporidium parvum* Oocysts by PCR. *Applied and Environmental Microbiology* **61**: 4514-4516.

Widmer, G., Orbach, E., and Tzipori, S. (1999) Beta-tubulin mRNA as a Marker of *Cryptosporidium parvum* Oocyst Viability. *Applied and Environmental Microbiology* **65**: 1584-1588.