

LOW PRESSURE UV INACTIVATION OF *CRYPTOSPORIDIUM PARVUM* AND *GIARDIA LAMBLIA* BASED ON INFECTIVITY ASSAYS AND DNA REPAIR OF UV-IRRADIATED *CRYPTOSPORIDIUM PARVUM* OOCYSTS.

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INTRODUCTION

Cryptosporidium parvum and *Giardia lamblia* are two of the most important waterborne pathogens. They are ubiquitous in surface and source waters (LeChevallier et al., 1995) and very resistant to conventional water treatment processes (Gibson III et al., 1998). A properly operated conventional filtration system achieves some removal of this microorganism (Gibson III et al., 1998), but most chemical disinfection processes are incapable of achieving appreciable inactivation of these microorganisms at practical doses and contact times (Sobsey, 1989, Clark, 1993). Previous studies based on the in vitro viability assays of excystation and vital dye staining suggested that *C. parvum* oocysts and *G. lamblia* cysts are also very resistant to UV radiation (Ransome et al, 1993, Campbell et al, 1995, Rice et al, 1981). However, recent studies using in vivo animal infectivity assays indicate that polychromatic UV radiation extensively inactivates these microorganisms at relatively low doses (Clancy et al, 1998, Finch et al, 1999). A significant number of water and wastewater treatment plants utilizing UV disinfection, however, employ monochromatic, low-pressure (LP) UV radiation instead of polychromatic, MP UV radiation as a radiation source. Therefore, we determined the kinetics and extent of inactivation of *C. parvum* oocyst infectivity for MDCK cell cultures and *G. lamblia* cyst infectivity for Mongolian gerbils by various doses of monochromatic, LP UV radiation.

Despite the recently recognized promise of UV disinfection, there are still several issues which must be addressed before widespread use of this technology as a primary disinfectant to achieve microbially-acceptable drinking water. One of the most important issues is the potential for *Cryptosporidium* oocysts to repair their UV-damaged DNA. Although the exact mechanism of inactivation of *Cryptosporidium* by UV radiation is not known, it is generally accepted that

nucleic acids, both DNA and RNA, are the primary targets of UV radiation (Jagger, 1967). It is possible that the high sensitivity of *C. parvum* oocysts to UV radiation may be due to their high DNA content. The *C. parvum* genome has five chromosomes ranging in size from 1,400 kb to over 3300 kb (Lally et al, 1992). If DNA is one of the primary targets of UV radiation in *C. parvum*, an important factor to consider is DNA repair. It is well known that some of the health-related microorganisms in water do have one or more of DNA repair pathways, including most of indicator bacteria (Whitby et al, 1984, Harris et al, 1987), and at least some pathogenic bacteria (U. S. EPA, 1986, Das et al, 1981). However, it is not known if a coccidian protozoan such as *C. parvum* has any of DNA repair pathways. Therefore, we determined the potential for and magnitude of DNA repair in UV-irradiated *C. parvum* oocysts

MATERIALS AND METHODS

Parasites

Cryptosporidium parvum oocysts (Iowa strain) were purchased from Pat Mason, Pleasant Hills Farm, Troy, Idaho. Shed oocysts collected daily from experimentally infected 3-day old calves were screened to remove large debris and hair and then purified and dispersed by processing through discontinuous sucrose gradients, followed by cesium chloride (CsCl) gradients (1.15 g/ml, 1.15 specific gravity). Oocysts recovered from CsCl gradients were washed in phosphate buffered saline (PBS, pH 7.2), resuspended in buffer solution containing antibiotics and stored at 4 °C. *Giardia lamblia* cysts were purchased from Parasitology Research Labs, Neosho, Missouri. Shed cysts collected from experimentally infected Mongolian gerbils and were screened to remove large debris, mixed with zinc sulfate (ZnSO₄, 1.2 specific gravity) solution, and centrifuged at 1,500 rpm for 5 minutes. Cysts recovered from supernatant were washed well with distilled water, resuspended in buffer solution containing antibiotics and stored at 4 °C.

Low-pressure UV radiation system and radiometry

A low-pressure, collimated beam UV apparatus consisting of two 15 Watt germicidal lamps which produced nearly monochromatic UV radiation at 254 nm was used. The radiation was collimated through a circular opening to provide incident radiation normal to the surface of the test suspension in a 60 X 15 mm cell-culture petri dish. UV irradiance was measured with an International Light IL500 radiometer at a wavelength of 254 nm. The radiometer had been factory-calibrated, traceable to NIST standards, just prior to this study.

Petri factor and dose determinations

Because radiation from a collimated beam is not actually 100 percent collimated, the irradiance measured at the center of the surface area of the petri dish was corrected to represent the average irradiance across the petri dish surface area. A spreadsheet for calculation of the petri factor and determination of average incident irradiance across the surface area of the liquid in the petri dish was developed to facilitate the determination of exposure time necessary to achieve a given UV dose. The dose was computed as the product of average radiation irradiance and time (in seconds) after adjusting the average radiation irradiance according to the absorbance of the suspension. The necessary exposure time was calculated by dividing the desired UV dose by the measured UV irradiance.

Experimental protocol for UV disinfection experiments

Purified stocks of *Cryptosporidium parvum* oocysts or *Giardia lamblia* cysts were diluted in phosphate buffered saline (PBS) to give final concentrations of $\sim 10^6$ /ml. Aliquots of 5 ml in 60 X 15 mm cell culture (petri) dishes were irradiated with the aforementioned collimated beam UV source while stirring the samples slowly on a magnetic stir plate at room temperature (23-25 °C). After predetermined exposure times, samples were removed from the UV irradiation system and diluted serially 10-fold for subsequent microbial assays.

Experimental protocol for DNA repair experiments

Five samples of *Cryptosporidium parvum* oocysts, 5-ml each in 60 mm diameter petri dishes, were exposed to a low dose of UV irradiation and then wrapped with aluminum foil immediately after UV exposure for DNA repair experiments. One sample was kept at 4 °C as an experimental control. The other four samples were transferred to either a 25 °C or 37 °C incubator. One of the two dishes at each temperature was illuminated by a 15 Watt fluorescent lamp at distances of 25-50 cm with slow stirring (light repair) and the other was just stirred while kept wrapped with aluminum foil (dark repair). Two experimental conditions for DNA repair were tested: one based on the conditions for bacterial DNA repair (37 °C for 1-2 hours) and the other based on conditions for mammalian cell DNA repair (25 °C for 2-4 hours). After the incubation time, the samples were immediately serially-diluted and inoculated on confluent MDCK cell cultures for infectivity assay.

Microbial Assays

Cryptosporidium parvum infectivity assays were done in Madin-Darby Canine Kidney (MDCK) cell cultures (ATCC CCL 34). MDCK cells were maintained in Eagle's Dulbecco's Modified Eagles Medium (DMEM/F1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 15 mM HEPES. For assays, cells were seeded at 7.5×10^4 cells per double-chambered Lab Tech Chamber Slide (Nalge Nunc International) in a volume of 1.5 ml of Ultraculture™ serum-free medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 250 µg/ml kanamycin, 50µg/ml gentamicin, 150 µg/ml mycostatin and 2 mM L-glutamine. Cultures were incubated at 37°C in a 5% CO₂ environment for four days. Four-day-old MDCK cell monolayers in slide chambers were inoculated with duplicate 300 µl volumes of undiluted or serially 10-fold diluted oocyst suspensions, and incubated for three hours at 37°C to promote excystation and initial cell infectivity. After incubation, the cell monolayers were washed with PBS, 1.5 ml of fresh Ultraculture medium was added to the chambers and the cells were incubated for 48 hours. After incubation, cell layers were washed with PBS, fixed in Bouin's solution (Sigma, St. Louis, MO.), decolorized, and blocked with 1% bovine serum albumin (BSA) in PBS. Cell layers were inoculated with 300 µl of C3-C3 antibody specific for *C. parvum* living stages that had been conjugated with the red fluorochrome Cy3 or with apple green fluorescein isothiocyanate. Antibody-treated cell layers were incubated for 90 minutes in the dark at room temperature, after which the labeled cell cultures were washed, mounted under coverslips and observed microscopically. Labeled life stages were assessed by UV illuminated epifluorescent microscopy using either a 25X or 40X objective. Infectivity was assayed by a quantal approach in which the presence or absence of living stages (sexual gamonts and asexual meronts) was scored in 50-100 sequential, non-overlapping fields. Any field containing a fluorescent developmental stage of *Cryptosporidium* received a positive score. A negative score was assigned to those fields that contained no fluorescent life stages. The number of infected and

uninfected test units fields was scored and recorded. Oocyst infectivity titer was calculated as a Most Probable Number (MPN) from the Thomas Equation using the dilutions where only some of the fields observed were infected.

Giardia lamblia infectivity assays were done in 8- to 10-week old female Mongolian gerbils (*Meriones unguiculatus*). The gerbils were bought from Charles River, Canada (St. Constant, Qc). At least 10 days before experimental infection, the animals were treated once with a solution (20 mg per gerbil) of metronidazole (Flagyl; Rhone Poulenc, Montreal, Qc) which was administered by gavage. This treatment ensured that the gerbils were free from all previous intestinal infections (including *Giardia*), as demonstrated by three consecutive examinations of feces. Feces were collected daily starting 3 days after infection and lasting until day 25. This period of collection was chosen because it represents the latent, the acute, and the elimination phase of *Giardia* infection in gerbils (Belosevic et al, 1983). The total number of cysts released in a 2-hr fecal collection (10 a.m. to 12 p.m.) by individual gerbils was determined by the sucrose flotation method as described previously (Belosevic and Faubert, 1983). The presence of trophozoites in the small intestine was determined at day 15 after infection and at the end of the experiment after the gerbils were killed in a CO₂ gas chamber. For the day 15 intestinal examination, one gerbil from each group was sacrificed. The small intestine was slit longitudinally and divided in small segments and placed in 20 ml of phosphate-buffered saline (pH 7.2). After incubation for 2.5 hr at 37 °C in a shaking (100 cycles/min) water bath, the number of trophozoites recovered was determined microscopically with use of a hemocytometer. The infectivity titers of *G. lamblia* cysts were calculated as a most probable number (MPN) based on the presence or absence of *G. lamblia* cysts and trophozoites in individual animals inoculated with sample dilutions out of a total of five animals per sample dilution.

Data presentation

For each microorganism, the concentrations in control samples were computed and taken as N₀, the initial concentrations. For each test sample, the average concentrations of each microorganism were computed. The proportions of initial microorganisms remaining at each dose (d) were computed by dividing the concentration at each dose (N_d) by the initial concentration (N₀). These values were then log₁₀-transformed (log₁₀ (N_d/N₀)), and the values of replicate experiments were averaged. The data for log₁₀ (N_d/N₀) of averaged values of replicate experiments were then paired with the data for dose (d) and plotted.

RESULTS

Figure 1 shows the average reduction kinetics of *Cryptosporidium parvum* infectivity and *Giardia lamblia* infectivity by several different doses of monochromatic, low-pressure UV radiation applied to the test microorganisms in phosphate buffered saline at room temperature. The reductions of *Cryptosporidium parvum* infectivity were very rapid and reached the detection limits of the infectivity assays (~ 3 log₁₀) within a dose of 3 mJ/cm². The reduction of *Giardia lamblia* cyst infectivity for gerbils was even more rapid and reached a detection limit of >~ 4 log₁₀ within a dose of 1 mJ/cm².

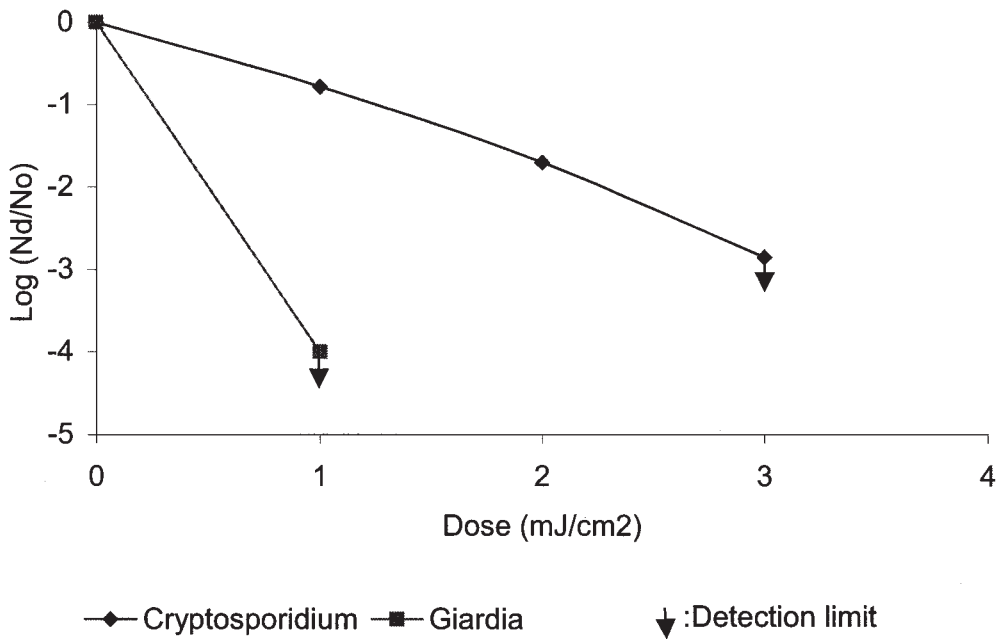


Figure 1. Inactivation kinetics of *Cryptosporidium parvum* and *Giardia lamblia* by monochromatic, low- pressure UV radiation

Table 1 shows repair of UV-irradiated *Cryptosporidium parvum* oocysts dosed with 1.23 mJ/cm² of LP-UV. The reduction of *C. parvum* oocyst infectivity by UV doses of 1.23 mJ/cm² was not recovered by exposing UV-irradiated oocysts to either light or dark repair conditions. Therefore, based on the extent of infectivity reduction, there was no evidence of either light or dark repair of DNA damage caused by UV irradiation of *C. parvum* oocysts.

Table 1. Repair of UV-irradiated *Cryptosporidium parvum* oocysts dosed with 1.23 mJ/cm² of LP-UV

Conditions	<i>Cryptosporidium parvum</i> reduction (-Log ₁₀ value)		
	Control	Dark	Light
37 °C, 1 hour	0.8	0.8	0.85
37 °C, 2 hour	0.8	1.7	1.0
25 °C, 2 hour	0.8	0.85	1.0
25 °C, 4 hour	0.7	1.4	0.9

DISCUSSION

The results of this study suggest that *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts are very sensitive to monochromatic low-pressure UV radiation. Appreciable inactivations of *C. parvum* oocysts and *G. lamblia* cysts appear to be achieved by practical doses of low-pressure UV radiation as well as by polychromatic medium-pressure UV radiation (Clancy et al, 1998, Finch et al, 1999). Previous research has shown that the 254 nm wavelength of low-pressure UV systems is one of the most microbiocidal wavelengths in the UV and visible light spectrum (Hollaender et al, 1944). It is likely that this wavelength also is one of the most important one in the output spectra of medium-pressure UV radiation in terms of inactivation of *C. parvum* oocysts and *G. lamblia* cysts.

The results of this study show that the kinetics of inactivation of *Giardia lamblia* cysts appears to be first-order through at least a 4 log₁₀ (99.99 %) reduction. This is in contrast to the results of a previous study (Finch et al., 1999) reporting a “plateau” around 2.5 log₁₀ reduction for UV inactivation of *G. muris* cysts. It is not certain if this discrepancy is due to different *Giardia* species (*lamblia* vs. *muris*), the purity and physical state of the cysts, UV dosimetry conditions, the variability of animal infectivity assay, or other factors.

There is no apparent phenotypic evidence of either light or dark repair of UV-damaged DNA in *Cryptosporidium parvum* oocysts under the conditions tested (using both bacterial and mammalian DNA repair conditions). However, this does not necessarily mean that there is no potential for repair of UV-damaged DNA in *C. parvum* oocysts. Perhaps *C. parvum* oocysts have different DNA repair mechanisms which can be activated under different environmental conditions than those used in this study. More thorough biochemical and genetic investigations are needed on the presence of DNA repair enzymes in *C. parvum* using different reactivation conditions. Whether or not *C. parvum* oocysts can be reactivated by light or dark DNA repair conditions will aid in determining the required doses of low-pressure UV radiation needed to inactivate *C. parvum* oocysts in water and wastewater treatment systems.