Susceptibility of five strains of *Cryptosporidium parvum* oocysts to UV light

*Cryptosporidium* is recognized as an important and widely distributed enteric pathogen of young livestock and is common in a variety of mammals (Casemore et al, 1997). The life cycle may result in production of resistant oocysts that exhibit long survival times in the aquatic environment. Certain species, including *C. parvum* (Chappell et al, 1996; Dupont et al, 1995), *C. felis* (Caccio et al, 2002), *C. meleagridis* (Pedraza-Diaz et al, 2001), *C. baileyi* (Ditrich et al, 1991), *C. canis* (Fayer et al, 2001), and possibly others, have been classified as zoonotic agents and have been associated with severe diarrheal disease in humans, although *C. parvum* is the significant human pathogen. Chronic infection may occur in the immunocompromised population, where mortality rates as high as 33% have been observed (Kramer et al, 1996).

Under the proposed Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) (USEPA, 2003a), large surface water treatment facilities will have to perform source water characterization monitoring to determine treatment requirements specific to the system. Treatment options will include, but are not limited to, filtration and chemical disinfection. However, the oocysts are
marginally affected by chlorine-based chemical disinfectants (Finch et al, 1993; Korich et al, 1990) and ozone (AWWARF, 1997) at practicable application levels. In addition, concern over the by-products of chemical disinfection has prompted investigations of alternative disinfection methodologies. Although utilities will be permitted to implement ultraviolet (UV) disinfection for regulatory compliance, they will be compelled to meet minimum dose requirements based on the known UV dose–response of pathogens such as Cryptosporidium. A draft UV guidance manual (USEPA, 2003b) prepared by the US Environmental Protection Agency (USEPA) in conjunction with industry stakeholders, was made available in June 2003 and provides additional guidance for utilities intent on implementing UV disinfection for control of Cryptosporidium and other waterborne pathogens.

UV BACKGROUND

UV has long been recognized as an effective disinfectant of bacteria (Zelle & Hollaender, 1955; Sharp, 1939) and viruses (Wilson et al, 1992; Chang et al, 1985; Hill et al, 1970). UV promotes the formation of pyrimidine dimers within the nucleic acids of irradiated cells (Mitchell & Nairn, 1989). The extent of inactivation is a function of characteristics specific to the organism and the UV dose, which is the product of the incident irradiance expressed as mW/cm² and the time (in seconds) of exposure. This product is expressed as mW·s/cm² and is equivalent to mJ/cm². The dose requirements for 4-log₁₀ (99.99%) inactivation of microorganisms range from 3–10 mJ/cm² for vegetative bacteria (Wilson et al, 1992; Chang et al, 1985) to 20–50 mJ/cm² for a number of human enteric viruses (Wilson et al, 1992).

UV and drinking water treatment. UV technology has been applied to drinking water for control of bacteria and viruses in Europe (Sommer et al, 1998) and in wastewater in the United States since the 1950s. Until recently, however, it had not been accepted as an effective disinfectant for control of Cryptosporidium. Early research using in vitro methods (excystation and vital dye stains) to assess oocyst viability following UV treatment suggested that a UV dose of 120 mJ/cm² would achieve only 2-log₁₀ inactivation (Ransome et al, 1993) and that doses >8,700 mJ/cm² were necessary for 3-log₁₀ inactivation (Campbell et al, 1995).

More recent investigations, using animal infectivity to assess oocyst survival, have shown UV to be an effective disinfectant of C. parvum oocysts, achieving 2-log₁₀ inac-
tivation at 2 mJ/cm² (Shin et al, 2001) and 4-log₁₀ inactivation at 8 mJ/cm² (Clancy et al, 2000). Several other studies corroborated these findings that C. parvum is susceptible to inactivation by low doses of UV light (Mackey et al, 2002; Craik et al, 2001; Mofidi et al, 2001; Hargy et al, 2000; Bukhari et al, 1999). Each of these studies used Iowa strain oocysts. Any assessment of the applicability of UV to drinking water treatment facilities for disinfection of Cryptosporidium oocysts must characterize the UV dose–response of Cryptosporidium strains (other than the Iowa isolate of C. parvum) that are infectious to humans.

**Five strains studied.** The current study examined the response to UV doses of 5–40 mJ/cm² of five strains of C. parvum isolated from different sources across North America and Europe. Cryptosporidium strains were obtained from Scotland (Moredun and Glasgow strains) and the United States (Iowa, Maine, and Texas A&M University [TAMU] strains). Previous genotyping studies of these isolates indicated that they belong to C. parvum genotype 2 and are capable of causing infection in both humans and some animals (Rochelle et al, 1999; Spano et al, 1998; Carraway et al, 1997; Peng et al, 1997; Ortega et al, 1991).

**Origin of Iowa strain.** The Iowa strain of C. parvum is regularly maintained at the Sterling Parasitology Laboratory at the University of Arizona in Tucson and was originally obtained from Harley Moon of the National Animal Disease Center in Ames, Iowa. It has been used to determine the C. parvum infectious dose–response in neonatal mice (Korich et al, 2000; Finch et al, 1993; Korich et al, 1990) and to characterize the sensitivity of Cryptosporidium to various disinfectants including chlorine, chlorine dioxide, monochloramine (Korich et al, 1990), ozone (Bukhari et al, 2000; AWWARF, 1997; Finch et al, 1993), and UV light (Clancy et al, 2000; Bukhari et al, 1999; Clancy et al, 1998). In addition, the Iowa isolate has been used in various studies examining infection characteristics and the immune response of Cryptosporidium in controlled experiments with healthy human volunteers (Okhuysen et al, 1998; Chappell et al, 1996; Dupont et al, 1995). It has also been used for infectivity studies in gamma interferon knockout mice (Mead & You, 1998).

**Origin of Moredun strain.** The Moredun strain was obtained from Steve Wright of the Moredun Research Institute in Penicuk, Scotland. It was originally isolated from a red deer calf (Cervus elaphus) (Blewett, 1989) and has been passaged in calves and lambs for approximately 12 years. This isolate has been used in environmental survival studies (Robertson et al, 1992), infectivity studies (Bukhari & Smith, 1997; Bukhari et al, 1995), biochemical studies (Awad-el-Karim et al, 1998; Nina et al, 1992), volunteer studies, and immunological characterization studies (Ortega-Mora & Wright, 1994). This isolate is used in the quality assurance/quality control approach instigated by the United Kingdom Drinking Water Inspectorate for continuous monitoring programs for finished waters (UKDWI, 2003).

**Origin of TAMU strain.** The TAMU strain was obtained from a veterinary student who was exposed to oocysts during necropsy of an infected foal. The isolate has since been propagated in calves at the Sterling Parasitology Laboratory. Dose–response studies indicate that the
ID₀₅₀ (the dose required to infect 50% of exposed individuals) for the TAMU isolate in healthy volunteers (ID₀₅₀ = 9) may be approximately 10-fold lower than the ID₀₅₀ of the prototype Iowa isolate (ID₀₅₀ = 87) (Okhuysen et al, 1998) and perhaps 100-fold lower than the infective dose of the Ungar C. parvum isolate (ID₀₅₀ = 1,042) (Okhuysen et al, 1999).

**Origin of Maine strain.** The Maine strain was obtained from Michael Arrowood of the Centers for Disease Control and Prevention in Atlanta, Ga. This isolate was responsible for an outbreak of cryptosporidiosis traced to contaminated apple cider (Millard et al, 1994). Oocysts were isolated directly from the cider, the press used for preparing the cider, and a calf stool specimen from the farm that supplied the apples. Experimental infection studies showed that this isolate is capable of infecting mice, calf, and human hosts; it has been characterized as a genotype 2 isolate using the TRAP-C2 polymorphic marker (Peng et al, 1997).

**Origin of Glasgow strain.** The Glasgow strain was received from Huw Smith at the Scottish Parasite Diagnostic Laboratory in Glasgow, Scotland. These oocysts were purified from feces of human patients involved in a waterborne outbreak in Glasgow (Smith, 2001).

**MATERIALS AND METHODS**

**Microorganism strains and growth conditions.** The C. parvum oocysts used in this study were maintained and propagated in the following manner. Within 4–12 h of birth, Holstein calves were fed 2 × 10⁸ oocysts suspended in sterile water. The calves were given four pints of colostrums 1–2 h following inoculation. Calves also received prophylactic doses of oral rotavirus, coronavirus, and *Escherichia coli* vaccines. Subsequently, the calves were maintained on a diet providing 2,200 kcal/day supplied by a combination of milk replacer, nonfat milk powder, and electrolyte mix.

The total daily fecal output from the infected calves was collected, screened through sieves, and concentrated by centrifugation. Oocysts were isolated from the feces by discontinuous sucrose gradients followed by microcentrifuge-scale cesium chloride gradients (Arrowood & Donaldson, 1996; Arrowood & Sterling, 1987). The purified oocysts were stored at 4°C in 0.01% polyoxyethylene-sorbitan monolaurate solution¹ containing 100 U of penicillin, 100 µg of streptomycin, and 100 µg of gentamicin/mL to retard bacterial growth. These oocysts were

### TABLE 2 Irradiance calculation factors for *Escheria coli*, MS2, and *Cryptosporidium parvum*

| Organism Type and Identification | UV₂₅₄* Absorbance/1 cm | Petri Factor |
|--------------------------------|------------------------|--------------|
| E. coli 1                       | 0.204                  | 0.991        |
| E. coli 2                       | 0.305                  | 0.981        |
| E. coli 3                       | 0.180                  | 0.997        |
| E. coli 4                       | 0.316                  | 0.975        |
| MS2 1                           | 0.012                  | 0.991        |
| MS2 2                           | 0.026                  | 0.981        |
| MS2 3                           | 0.215                  | 0.971        |
| MS2 4                           | 0.212                  | 0.975        |
| C. parvum Iowa                  | 0.091                  | 0.976        |
| C. parvum Moredun               | 0.175                  | 0.971        |
| C. parvum TAMU†                 | 0.386                  | 0.981        |
| C. parvum Maine                 | 0.211                  | 0.991        |
| C. parvum Glasgow               | 0.154                  | 0.982        |

*UV₂₅₄*—ultraviolet light at 254 nm

¹TAMU—Texas A&M University

An analyst prepares dilutions of coliphage MS2 for enumeration of surviving fraction following ultraviolet irradiation.

Laboratory in Glasgow, Scotland. These oocysts were purified from feces of human patients involved in a waterborne outbreak in Glasgow (Smith, 2001).
Excystation efficiency was measured prior to each trial (Robertson et al., 1993). Oocysts were deemed acceptable for use only if the in vitro excystation method described by Robertson and colleagues (1993) and modified by Korich and colleagues (2000). This model relates the proportion of mice infected to the number of oocysts inoculated. Briefly, response logit (RL) was calculated as the natural logarithm of the proportion of animals infected divided by one minus the proportion of animals infected. That is,

\[ RL = \ln \left( \frac{P}{1-P} \right) \]  

where \( P \) is the proportion of animals infected at a given oocyst dose.

The untreated oocyst dose–response curves were obtained by performing a least-squares regression of RL on the logarithm of the number of untreated oocysts in each dose. The regression analysis produced equations of the form

\[ y = mx + b \]  

in which \( y \) is the RL, \( x \) is the logarithm of the oocyst dose, and \( b \) is the \( y \) intercept. Table 1 lists these equations for the five \( C. parvum \) strains investigated in this research.

The equations used in this study were based on dose–response data obtained by the authors as well as data from a concurrent study conducted with Rochelle and co-workers (2002). These regression equations were used to calculate the number of infective oocysts in each dose of UV-treated oocysts. This was done by substituting the RL derived from UV-treated oocysts into the regression equations and solving for dose. The regression equations were also used to determine the ID50 by setting the RL equal to zero and solving for dose. Log-inactivation levels were determined by subtracting the log of the number of infectious oocysts prior to UV exposure (\( D_0 \)) from the log of the number of infectious oocysts in the treated dose (\( D \)). That is,

\[ \text{log inactivation} = \log D - \log D_0 \]  

For the instances in which no infections were observed in mice fed with UV-treated oocysts, minimum \( \log_{10} \) inactivations were inferred using the endpoint sensitivity of the assay (1 infection per litter) and were reported as “greater than” values. Untreated control oocysts were evaluated for each test by comparing their dose–response to the appropriate dose–response curve. Controls were considered satisfactory as long as the dose–response fell within the 80% prediction bands of each curve.

**Male-specific coliphage.** Coliphage MS2 was propagated in *E. coli* F-amp according to the double agar layer described by Adams (1959). *E. coli* F-amp was cultured overnight in trypticase soy broth (TSB) containing ampicillin and streptomycin at 36°C, transferred to fresh TSB,
and incubated for an additional 4 h at 36°C. Serial tenfold dilutions of bacteriophage stocks were prepared in phosphate-buffered water and were added to melted top agar tubes containing 0.7% agar and 0.5% sodium chloride. Then 100 µL each of log-phase bacteria and coliphage were added to top agar tubes; samples were mixed and poured over nutrient bottom agar plates containing 1.1% nutrient agar and 0.5% sodium chloride. Following overnight incubation at 37°C, the upper soft agar layer from confluent lysis plates was harvested with 5 mL phosphate-buffered solution and supplemented with an equal volume of chloroform. Samples were vortexed for 60 s and then centrifuged at 5,000 × g for 15 min. Viruses in the aqueous supernatant were recovered, filtered through membranes pretreated with 0.1% polyoxyethylenesorbitan monooxolate, and stored at 4°C until day of use. Unused samples were discarded after 30 days.

**E. coli.** Cultures of *E. coli* were prepared by inoculating 250 mL sterile TSB and incubating on a shaker apparatus at 35°C for approximately 18 h. This resulted in a late log-phase culture with an *E. coli* concentration of approximately 1–3 × 10^8 cfu/mL. Cultures were harvested and washed one time and resuspended in deionized water for immediate use.

**UV irradiation.** The UV source was a low-pressure mercury vapor lamp. Low-pressure lamps emit nearly monochromatic UV radiation at 254 nm, which can be precisely monitored with a calibrated sensor. The 254-nm wavelength closely corresponds with peak wavelength of germicidal UV near 260 nm. This lamp was housed above a solenoid-operated shutter connected to a digital timer. When the shutter was opened, light from the lamp passed through a 33 cm (13 in.) collimating tube to irradiate the test organisms that were suspended in a 6 cm (2.4 in.) diameter petri dish. Prior to irradiations on each day of testing, the lamp was allowed to warm up for at least 30 min.

The UV incident to the surface of the petri dish was measured using a radiometer and detector calibrated at 254 nm. The incident irradiation across the surface of the petri dish was measured at 5 mm (0.2 in.) intervals along an x–y grid originating at the center of the dish. The overall irradiance distribution, or petri factor, was determined relative to the center reading. This value was then used in the calculation of average irradiation incident to the water surface (Bolton & Linden, 2003). Factors influencing average irradiation to the entire volume included reflection from the water surface, depth of the water, and UV absorbance of the inoculated test water. The last was measured at 254 nm by spectrophotometry. Table 2 shows the UV_{254} absorbance in 1 cm (0.4 in.) and petri factor for each test. UV dosage was defined as the average irradiation in the exposure solution multiplied by the exposure time.

Irradiations of suspensions of each surrogate organism were made in duplicate across a range of exposure times. *E. coli* was exposed to UV doses of 0, 2.5, 5.0, 7.5, and 10 mJ/cm^2^ and MS2 to doses of 0, 10, 20, 30, 40, and 50 mJ/cm^2^.

Test organisms were suspended in 15 mL of deionized water in 6 cm (2.4 in.) diameter petri dishes with a 12 mm (0.5 in.) stir bar. A preenumerated stock suspension containing 5 × 10^7 live *Cryptosporidium* oocysts of the appropriate strain in 15 mL of deionized water was vortexed for 30 s and added to the continuously stirred petri dish. After 1 min, the UV lamp shutter was opened and the suspension was irradiated for the predetermined length of time. A control dose (0 mJ/cm^2) was run simultaneously with the irradiation test for the highest dose level for each strain. Controls were dishes containing oocysts suspended and stirred in the test water in the absence of UV. Other research has found no photoreactivation with *Cryptosporidium* (Shin et al, 2001; Rochelle et al, 1999), and no precautions were taken to avoid white light exposure.
RESULTS

Confirmation of UV dose. The dose–response curves generated for *E. coli* and coliphage MS2 are shown in Figures 1 and 2, respectively, along with the UV dose–responses of these organisms derived from the literature. The inactivation of *E. coli* by 2.5 and 5 mJ/cm² exceeded that predicted by Sommer and colleagues (1998) on two test dates. Tests using exposure times expected to provide 5 mJ/cm² achieved inactivation levels predicted for 7.5 mJ/cm² in the reference. Although this constitutes a 50% difference, the results do indicate that the lower target doses were not overapplied by orders of magnitude.

Notably, the response of the MS2 to UV doses of 10–50 mJ/cm² fell within the range specified in the USEPA draft UV guidance manual (USEPA, 2003b); the response to UV doses of 10–40 mJ/cm² fell within the range suggested by the National Water Research Institute and AWWA Research Foundation guidelines (NWRI/ AWWARF, 2003). These dose comparisons support the reliability of the radiometer irradiance readings and offer confidence that the reported UV doses were applied to the *Cryptosporidium* oocysts.

UV dose–response. Table 3 and Figure 3 show the results of UV treatment on the oocyst strains tested. UV doses of 10 and 40 mJ/cm² were selected originally for all strains. Because other research has provided a large body of data for the Iowa strain in this UV dose range (Craik et al, 2001; Clancy et al, 2000; Bukhari et al, 1999; Finch & Belosevic, 1999), a reduced dosage range was evaluated in this study (2–4 mJ/cm²) to assess the effect of low UV dose for that strain. Shin and co-workers (2001) showed that a low-pressure UV dose of 3 mJ/cm² resulted in 2.6-log₁₀ inactivation of the Iowa strain of *C. parvum* using cell culture to measure inactivation. The results in the current study showed good agreement with those data, with 3.2-log₁₀ inactivation at a UV dose of 2 mJ/cm² and 4.1-log₁₀ inactivation at 4 mJ/cm². The inactivation of the Moredun strain could not be precisely measured; UV doses of 10 and 40 mJ/cm²

| Parameter | Iowa | More|edun | TAMU† | Maine | Glasgow |
|-----------|------|-----|------|-------|-------|--------|
| UV dose—mJ/cm² | 0 | 2 | 4 | 3 | 10 | 40 | 0 | 10 | 40 | 0 | 5 | 20 | 0 | 5 | 20 |
| Number of oocysts/mouse | 1.5 × 10⁵ | 1.1 × 10⁴ | 9.4 × 10³ | 1.3 × 10³ | 9.0 × 10² | 1.5 × 10² | 1.2 × 10¹ | 1.4 × 10⁰ | 1.1 × 10⁰ | 8.1 × 10⁰ | 1.1 × 10⁰ | 9.6 × 10⁰ | 1.1 × 10⁰ | 1.0 × 10⁰ | 1.0 × 10⁰ |
| Number of mice | 12 | 23 | 22 | 20 | 21 | 23 | 23 | 26 | 35 | 23 | 24 | 24 | 24 | 23 | 24 |
| Number of mice infected | 7 | 1 | 1 | 8 | 0 | 0 | 22 | 4 | 0 | 20 | 7 | 3 | 2 | 2 | 2 | 0 |
| Percent of mice infected | 58.3 | 4.4 | 4.6 | 40 | <4.8 | <4.4 | 95.7 | 15.4 | <2.9 | 87.0 | 29.2 | 12.5 | 91.7 | 4.4 | <4.2 |
| Number of infective oocysts | 92 | 7 | 7 | 19 | <2 | <2 | 117 | 9 | <4 | 414 | 6 | 1 | 174 | 2 | <2 |
| Log₁₀ inactivation | 0.0 | 3.2 | 4.1 | 0.8 | >5.6 | >4.9 | 0.0 | 5.2 | >5.5 | 0.0 | 4.3 | 5.9 | 0.0 | 5.6 | >5.7 |

*UV—ultraviolet
†TAMU—Texas A&M University

![Figure 3](image-url)
resulted in complete loss of oocyst infectivity, yielding a value of at least 5.6-log$_{10}$ inactivation at 10 mJ/cm$^2$. The TAMU strain showed $>5.5$-log$_{10}$ inactivation at 40 mJ/cm$^2$ with a measured 5.2-log$_{10}$ inactivation at 10 mJ/cm$^2$.

After noting the consistent high susceptibility of the Iowa, TAMU, and Moredun strains, the authors decided to use lower UV doses (5 and 20 mJ/cm$^2$) for the two remaining strains tested in an effort to avoid the use of assay detection limits. The Glasgow strain showed 5.6-log$_{10}$ inactivation at 5 mJ/cm$^2$, whereas the Maine strain showed 5.9-log$_{10}$ inactivation at 20 mJ/cm$^2$ and 4.3-log$_{10}$ inactivation at 5 mJ/cm$^2$. A UV light dose of 10 mJ/cm$^2$ achieved at least 4-log$_{10}$ inactivation of all strains evaluated.

**DISCUSSION**

This study evaluated the response of five strains of *C. parvum* oocysts to UV light. Prior to this research, only the Iowa strain had been investigated for its response to UV light. Because the effectiveness of UV technologies for the control of *Cryptosporidium* in drinking water has been recognized only recently (AWWARF, 2000; Clancy et al, 2000; Bukhari et al, 1999; Clancy et al, 1998), questions have arisen regarding the universality of the effect of UV on other oocyst strains infective to humans. Although these questions have not been answered for other chemical disinfectants such as ozone or chlorine dioxide, this study was designed to answer these questions with regard to UV.

Results showed that infectivity of all five strains tested was reduced by at least 4 log$_{10}$ or 99.99% at a UV dose $\leq$10 mJ/cm$^2$. In each trial, the UV irradiance measurements obtained by radiometry were verified by examining the response of organisms of known UV response—*E. coli* and coliphage MS2—with the test apparatus and methods and comparing the inactivation achieved against reference data.

Iowa strain oocysts were subjected to lower UV doses of 2 and 4 mJ/cm$^2$ in order to better characterize that strain’s response to low UV dose. Inactivations of 3.2 and 4.2 log$_{10}$ were achieved at these doses, indicating that *Cryptosporidium* is more susceptible to UV inactivation than many human enteric viruses and some enteric bacteria. These results apply only to the UV response of genotype 2 strains of *C. parvum*, which are capable of infecting both humans and some animals, and do not rule out the existence of some strain(s) exhibiting greater resistance to UV. However, the results do expand the understanding of the UV dose–response of *Cryptosporidium* oocysts from a single strain to five and bolster confidence in UV technology as an effective treatment of drinking water for disinfection of *Cryptosporidium*. Future research targeting the characterization of the UV response of *Cryptosporidium* should consider genotype 1 isolates as well.

UV doses as high as 40 mJ/cm$^2$ were evaluated in preliminary experiments because this dose was originally considered as a potential requirement for treating drinking water for *Cryptosporidium* control in the United States. Currently, UV dose requirements for drinking water disinfection have not been mandated by the federal government. However, USEPA is considering doses that may be $<12$ mJ/cm$^2$ to achieve 3-log credit for control of *Cryptosporidium* (Schmelling, 2002). In Austria (Austrian Standards Institute, 2001) and Germany (DVGW, 1997), doses of 40 mJ/cm$^2$ are required when UV disinfection of drinking water is practiced. USEPA is expected to promulgate the LT2ESWTR sometime in 2004, requiring up to 2.5-log$_{10}$ inactivation of *Cryptosporidium* in addition to the physical removal achieved by coagulation and filtration processes. The findings in this study support UV as a technology capable of achieving that level of inactivation. Economic analyses suggest that the...
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FOOTNOTES

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