Effect of the presence of coexisting substances on UV inactivation of *Cryptosporidium parvum* oocysts

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ABSTRACT

Three turbid substances were added to suspensions of *Cryptosporidium parvum* oocysts, and changes in the degree of ultraviolet (UV) inactivation of the *C. parvum* oocysts were evaluated by *in vitro* excystation and animal infectivity. Because the dose distribution of UV irradiation delivered to oocysts in turbid water may vary according to the degree of movement of the water, experiments were performed under vigorously mixed (completely mixed) conditions and stationary conditions. The estimated kinetic inactivation rate in the completely mixed system of turbid water (190 ntu) was 4.058 cm²/mJ, which was only 15% less than that in pure water (4.690 cm²/mJ). The estimated relative infectivity in the stationary system differed markedly from that obtained by regression of the plot of UV irradiation dose versus relative infectivity in pure water. Nevertheless, values of the relative infectivity obtained in animal experiments were nearly in agreement with the calculated values. In other words, despite decreases in the dose of UV delivered to oocysts under the influence of turbid substances, the efficacy of UV in inactivation of oocysts in turbid water was almost equal to that in pure water when expressed as per unit dose. Consequently, it became evident consequently that if the dose of UV delivered to oocysts was known, the degree of expected inactivation could be estimated. In addition, we added substances that adhere to the surface of *C. parvum* oocysts (humins and kaolins) to suspensions of *C. parvum* oocysts and studied the degree of UV inactivation. Adhesion of humins or kaolins particles to the oocyst wall resulted in a reduction in UV inactivation. However, the magnitude of the log₁₀ inactivation in a suspension containing humins or kaolins particles was only about 20% and 10%, respectively, less than that in pure water.

KEYWORDS

Cryptosporidium parvum, Ultraviolet, Inactivation, Coexisting substances, Adherent substances

INTRODUCTION

Cryptosporidiosis outbreaks arising from contaminated surface water supplies have been documented in many countries (Mackenzie *et al.*, 1994, Atherton *et al.*, 1995, Yamamoto *et al.*, 2000). It is believed that *Cryptosporidium parvum* oocysts are widespread in rivers; the concentration of *C. parvum* oocysts in source river waters is as high as 10⁴ oocysts/100 L (Rose *et al.*, 1991, LeChevallier *et al.*, 1991). Although coagulation-sedimentation and rapid sand filtration can reduce the number of *C. parvum* oocysts by 2 to 4 log (Niemiński *et al.*, 1995, Baudin and Laine, 1998, Hashimoto *et al.*, 2002), the expected rate of removal may not be sufficient to reduce the risk of infection to an acceptable level in cases where the source water is strongly contaminated with *C. parvum* oocysts. Chlorine has been used as a final disinfectant in many water supplies; however, *C. parvum* oocysts are insensitive to chlorine at the concentrations routinely used (Korich *et al.*, 1990, Gyurek *et al.*, 1997, Hirata *et al.*, 2000) and thus there is interest in developing an alternative and more effective disinfectant for inactivating such recalcitrant microorganisms.

Ultraviolet (UV) disinfection systems produce no hazardous by-products and are easy to maintain. UV irradiation is considered to be one of the more effective techniques for inactivating bacteria and viruses (Sobsey, 1989). Fewer studies have been conducted on the effects of UV irradiation on *C. parvum* than on bacteria and viruses because UV irradiation was not considered to be effective in inactivating *C. parvum* based on results of *in vitro* excystation (Ransome *et al.*, 1993). However, there is substantial evidence suggesting that UV irradiation at low doses can reduce the infectivity of *C. parvum* oocysts (Linden *et al.*, 2000, Clancy *et al.*, 2000, Shin *et al.*, 2001, Morita *et al.*, 2002,

Zimmer *et al.*, 2003).

One problem that needs to be addressed before adopting UV irradiation as a method for disinfecting water is the reduction in UV irradiation efficacy caused by the presence of turbid substances in water (Hirata *et al.*, 2002). In particular, substances that tend to adhere to the surface of *C. parvum* oocysts may reduce the effectiveness of disinfectants.

Therefore, in this study, we examined the effects of turbid substances in suspensions of *C. parvum* oocysts on the degree of UV inactivation of the oocysts using the animal infectivity assay. In addition, we evaluated the effects of humin or kaoline, which adhere to the surface of *C. parvum* oocysts, on the degree of UV inactivation of *C. parvum* oocysts using *in vitro* excystation and animal infectivity assays.

MATERIALS AND METHODS

Cryptosporidium parvum oocysts

The *C. parvum* HNJ-1 strain (human isolate by Dr. Iseki, Kanazawa University, Japan) that was passaged in SCID mice (C.B-17/Icr, CLEA Japan Inc, Tokyo, Japan) at the Research Institute of Biosciences, Azabu University, was used for this study. Fresh feces from several infected mice was placed in 500 mL of purified water, emulsified, and filtered through a 0.1-mm mesh nylon sieve. A filtrate containing *C. parvum* oocysts was obtained. The crude oocyst suspension (20 mL) was underlaid with sucrose solution (specific gravity of 1.100 at 20 °C) and centrifuged at $1,500 \times g$ at 4 °C for 15 minutes. The interface and upper layer were recovered, and diluted with 150 mM of pH 7.4 phosphate-buffered saline (PBS; 0.20 g of potassium dihydrogen phosphate, 0.20 g of potassium chloride, 1.15 g of disodium hydrogen phosphate and 8.0 g of sodium chloride in 1 liter of distilled water) containing 0.1% (v/v) Tween 80. The diluted solution was centrifuged at $1,500 \times g$ at 4 °C for 15 minutes. The precipitate was then diluted to 20 mL with PBS containing 0.1% (v/v) Tween 80. The suspension was underlaid with sucrose solution and centrifuged at $1,500 \times g$ at 4 °C for 15 minutes. The interface was recovered. Washing with PBS containing 0.1% (v/v) Tween 80 was performed four times in order to remove fecal matter. The stock of purified oocysts was stored in PBS at 4 °C and was used in experiments within 7 days.

UV irradiation of *C. parvum* oocysts in the presence of various substances

Three substances were added to suspensions of *C. parvum* oocysts and the efficacy of UV irradiation in inactivating the oocysts was studied. Substances added and conditions of UV irradiation in each of the 13 experiments are shown in Table 1.

Table 1. Substances that were placed in suspensions of *C. parvum* oocysts and conditions of UV irradiation.

Run	Added substance	Condition of added substance	Mixing system	Intensity** (mW/cm ²)	Time (s)	Dose (mJ/cm ²)	
						Surface***	Average****
1	Pure water		completely mixed	0.10	5	0.5	0.5
2			completely mixed	0.10	10	1.1	1.1
3			completely mixed	0.10	15	1.5	1.5
4			completely mixed	0.10	20	2.1	2.1
5	Concentrated substances from river water*	suspended uniformly	completely mixed	0.10	30	3.0	1.0
6		suspended uniformly	completely mixed	0.10	60	6.0	2.1
7		suspended uniformly	completely stationary	1.00	3	3.0	1.1
8		suspended uniformly	completely stationary	1.00	6	6.0	2.3
9	Humin	dissolved	completely mixed	0.20	77	15.4	1.5
10		suspended uniformly	completely mixed	0.20	37	7.4	1.5
11		adhered to oocysts	completely mixed	0.20	36	7.2	1.5
12	Kaoline	suspended uniformly	completely mixed	0.10	42	4.2	1.5
13		adhered to oocysts	completely mixed	0.10	36	3.6	1.5

*Concentrated with ultra-filtration membranes

**UV intensity at the surface of the suspension

***UV dose at the surface of the suspension

****Average UV dose from the surface to the bottom of the suspension

Turbid substances in river water were concentrated using hollow-fiber ultra-filtration (UF) membranes (90% cut off: 150,000 daltons; Daicel Membrane-Systems, Ltd., Osaka, Japan). The turbid substances were removed from the surface of the UF membranes by shaking with sterilized tap water. The removed substances were sterilized by chlorination (at 3,000 mg/L for 5 minutes) followed by UV irradiation (over 20 J/cm²) and were then diluted with sterilized tap water to 190 ntu. The turbidity of the suspension was determined by measuring the absorbance at 660 nm with a spectrophotometer (UV-150-2; Shimadzu Co., Tokyo, Japan). A purified suspension of *C. parvum* oocysts was placed into an open plastic tube with an inner diameter of 36 mm containing 30 mL of 150 mM PBS in order to produce a final concentration of 10⁶ oocysts per mL.

Because the dose distribution of UV irradiation delivered to oocysts in turbid water may vary according to the degree of movement of the water, experiments were performed under vigorously mixed and stationary conditions. Experiments were performed while varying the irradiation time and stirring conditions so as to produce either a completely mixed system or a completely stationary system. For UV irradiation of a completely mixed system, UV irradiation of low intensity (0.10 mW/cm²) was applied for a long period of time (30-60 s) to a suspension that was being stirred vigorously with a magnetic stirrer. In experiments that were performed in a stationary system, turbid water containing *C. parvum* oocysts was vigorously stirred for 3 min; immediately after the discontinuation of stirring, UV irradiation of high intensity (1.00 mW/cm²) was applied for a short period of time (3-6 s).

Humic acid and kaoline are substances that adhere to the surface of *C. parvum* oocysts. Humic acid (Wako Co., Osaka, Japan) was dissolved in a small volume of 0.01 M sodium hydroxide and diluted by 100 times. The dissolved humic acid solution was placed into three plastic tubes with an inner diameter of 36 mm. In the first tube, a suspension of *C. parvum* oocysts was added. In this tube, oocysts coexisted uniformly with dissolved humin. In the second tube, the pH of the dissolved humic acid solution was adjusted to 6 with hydrochloric acid, and a suspension of *C. parvum* oocysts was then added. In this tube, oocysts coexisted uniformly with the particulate form of humin. In the third tube, a suspension of *C. parvum* oocysts was added and then the pH was adjusted to 6 with hydrochloric acid; the oocysts were incubated for one hour, allowing humin molecules to adhere to the surface of the oocysts.

Kaoline (Wako Co., Osaka, Japan) was dispersed in 150 mM PBS and this solution was divided into two tubes with an inner diameter of 36 mm. In the first tube, a suspension of *C. parvum* oocysts was added and sodium pyrophosphate was then added as a dispersant. In this tube, the oocysts coexisted uniformly with kaoline. In the second tube, a suspension of *C. parvum* oocysts was added and this was incubated for one hour, allowing kaoline molecules to adhere to the surface of the oocysts.

The tubes containing *C. parvum* oocysts and the various substances were placed under a 5 W low-pressure mercury lamp (QCGL5W-14 97D; Iwasaki Electronic, Co., Ltd., Tokyo, Japan). The intensity of the UV irradiation at a wavelength of 254 nm was measured by a UV dose rate meter (UIT-150-A; Ushio INC., Tokyo, Japan). The UV irradiation dose was altered by controlling the exposure time using an electrical shutter.

Infectivity

Infectivity was determined in animal infectivity tests with SCID mice maintained in a specific pathogen-free area at the Research Institute of Biosciences, Azabu University. This study was approved by the Animal Research Committee of Azabu University. Five-week-old SCID mice were used in the experiments after a one-week period of conditioning for adaptation to the new cage. Mice were individually housed in cages and were given autoclave-sterilized food and water. Four to five mice were used per dilution. Five-fold serial dilutions of *C. parvum* suspensions containing various substances were made using sterilized tap water. A 0.5-mL aliquot was administered orally to each mouse. Mouse mortality at the early stages of the experiments was 2 out of 410 mice; no

mice died beyond one week after oral administration. Four weeks after oral administration, fresh feces were collected, suspended in 50 mL of purified water and emulsified using a vortex mixer. A 5-mL portion of this suspension was overlaid on 8 mL of sucrose solution and centrifuged at $1,500 \times g$ at 4°C for 15 minutes. The interface was recovered, and approximately 1 mL of the supernatant was filtered through a 25 mm cellulose acetate membrane disk filter (pore size, 0.8 micron). The filter was then stained using immunofluorescent antibodies against oocyst wall protein (Hydrofluoro Combo Kit, Strategic Diagnostics Inc., Newark, Del., USA) and was observed under an epi-fluorescent, differential contrast microscope (BX-60, Olympus, Tokyo, Japan) at a magnification of $400\times$ in order to determine the presence or absence of oocysts. The most probable number (MPN) of infection was calculated from oocyst-positive mice, using the MPN program developed by Hurley and Roscoe (1983). The relative infectivity (I_r) of each sample was calculated by equation (1):

$$I_r = \frac{\text{MPN}_a}{\text{MPN}_0} \quad (1)$$

where MPN_a is the most probable number of oocysts after UV irradiation, and MPN_0 is the most probable number of oocysts before UV irradiation.

***In vitro* excystation**

The viability of the *C. parvum* oocysts was determined by a modification of Woodmansee's method (Woodmansee, 1987). The original excystation protocol was slightly modified by the addition of a 5 minute preincubation in acidified Hank's balanced salt solution (pH 2.75) at 37°C prior to incubation for excystation. After the preincubation, oocysts were incubated in excystation medium containing 1.5% (w/v) taurocholic acid and 0.5% (w/v) trypsin at 37°C for 60 minutes. The numbers of intact oocysts (IO) before excystation treatment, partially excysted oocysts (PO), empty oocysts (EO) and free sporozoites (S) were enumerated under a phase-contrast microscope at a magnification of $400\times$. The excystation rate (V) was calculated by equation (2):

$$V (\%) = \frac{S/4}{\text{IO} + \text{PO} + \text{EO}} \quad (2)$$

The survival ratio (S_r) was calculated from equation (3):

$$S_r = \frac{V_a}{V_0} \quad (3)$$

Where, V_a is the excystation rate of the UV-irradiated sample and V_0 is the excystation rate of the control sample that was not subjected to UV irradiation.

RESULTS AND DISCUSSION

Effects of various substances on UV inactivation of *C. parvum* oocysts

If it is assumed that the UV intensity in a *C. parvum* oocyst suspension containing turbid substances decreases according to Lambert-Beer's law, the UV intensity [I_x (mW/cm^2)] at a given depth [x (cm)] would be calculated by equation (4):

$$I_x = I_0 \exp(-Ax) \quad (4)$$

where, I_0 is the UV intensity at the surface of the suspension (mW/cm^2) and A is the absorption coefficient of the suspension at a wavelength of 254 nm (cm^{-1}). The average intensity of UV irradiation from the surface to the bottom of the suspension [I_{av} (mW/cm^2)] is given by equation (5):

$$I_{av} = \frac{1}{R} \int_0^R I_x dx = \frac{1}{R} \int_0^R I_0 \exp(-Ax) dx \quad (5)$$

where, I_x is the UV intensity (mW/cm^2) at a given depth [x (cm)] and R is the thickness of the suspension (cm). The average UV irradiation dose [Dx (mJ/cm^2)] is therefore given by equation (6):

$$Dx = I_{av} \cdot t = \frac{t}{R} \int_0^R I_0 \exp(-Ax) dx \quad (6)$$

where, t is irradiation time (s).

Figure 1 shows a graph of the average dose of UV irradiation from the surface to the bottom of the suspension versus the relative infectivity of *C. parvum* oocysts (Run No. 1 to 6). The kinetic inactivation rate in turbid water was $4.058 \text{ cm}^2/\text{mJ}$, which was only 15% less than the kinetic inactivation rate in pure water ($4.690 \text{ cm}^2/\text{mJ}$). Based on this result, the effectiveness of UV irradiation in inactivating *C. parvum* oocysts in turbid water can be calculated by the following procedure. First, the average UV dose was calculated using equation (6) while considering the reduction in UV intensity in the suspension, and the obtained average UV dose was then applied to the relationship between the UV dose and relative infectivity in pure water.

The average relative infectivity (I_{rav}) in the completely stationary system was calculated by integrating the relative infectivity (I_{rx}) at a given depth [x (cm)] from the surface to the bottom of a suspension from equation (7):

$$I_{rav} = \frac{1}{R} \int_0^R I_{rx} dx \quad (7)$$

where R is the thickness of the suspension (cm). The relationship between the relative infectivity (I_{rx}) at a given depth [x (cm)] and the UV irradiation dose [Dx (mJ/cm^2)] is assumed to be given by equation (8), which was derived from the results of experiments performed in pure water:

$$I_{rx} = e^{-4.690 Dx} \quad (8)$$

Figure 2 shows the average UV irradiation dose, which was calculated from equation (6), versus the relative infectivity, which was calculated from equation (7). When the turbidity was 190 ntu and the depth of the suspension was 30 mm, the degree of UV beam penetration to the bottom of the suspension was expected to be less than 7%.

Figure 1. Plot of the observed relative infectivity of *C. parvum* oocysts versus the estimated average UV dose from the surface to the bottom of the oocyst suspension in the mixed system.

Symbols: $\circ$, pure water (observed);
 $\bullet$, 190 ntu (observed).

Figure 2. Plot of the observed or calculated relative infectivity of *C. parvum* oocysts versus the estimated average UV dose in the stationary system.

Symbols: $\circ$, pure water (observed);
 $\square$, 190 ntu (observed);
 -----, 190 ntu (calculated).

The estimated relative infectivity under such conditions markedly differed from that obtained in the equation expressing the relationship between UV irradiation dose and relative infectivity in pure water. The relative infectivity obtained in animal experiments (Run No. 1 to 4 and 7, 8) were nearly in agreement with those calculated using equation (7). It therefore appears that the effectiveness of UV irradiation in inactivating *C. parvum* oocysts in a stationary system can be calculated using equation (7).

Effect of adherent substances

Humins were mixed with suspensions of *C. parvum* oocysts by three different methods, and UV irradiation was applied. The $\log_{10}$ inactivation of *C. parvum* oocysts in excystation and animal infectivity upon UV irradiation is shown in Figures 3 and 4. In *C. parvum* oocysts in pure water, UV irradiation at an average dose of 230 mJ/cm^2 achieved 1.91 $\log_{10}$ inactivation of *C. parvum* oocysts in the *in vitro* excystation assay, and UV irradiation at an average dose of 1.50 mJ/cm^2 achieved 2.97 $\log_{10}$ inactivation in the mouse infectivity assay. Upon application of the respective UV doses to *C. parvum* oocyst suspensions containing dissolved humin, UV irradiation achieved similar levels of oocyst inactivation (1.98 $\log_{10}$ and 2.99 $\log_{10}$, respectively). Adhesion of humin particles to the oocyst wall tended to reduce the degree of inactivation. However, the magnitude of the $\log_{10}$ inactivation in suspensions containing humin particles was only approximately 20% less than that in pure water.

Our results indicate that for inactivation of *C. parvum* oocysts, the effectiveness of UV irradiation per unit dose in oocyst suspensions containing humin homogeneously mixed with oocysts was nearly as high as that in oocyst suspensions in pure water. This indicates that even though the UV beam passed through a turbid fluid, the effectiveness of the UV irradiation per unit dose remained unaltered. Our results also suggest that adhesion of humin to the oocyst wall had no marked shielding effect.

Figure 3. The $\log_{10}$ inactivation of *C. parvum* oocysts in excystation upon UV irradiation in the presence of humin. Bars indicate the means from two independent experiments, and the lines indicate the maximum and minimum values.

Figure 4. The $\log_{10}$ inactivation of *C. parvum* oocysts in animal infectivity upon UV irradiation in the presence of humin. Bars indicate the means from two independent experiments, and the lines indicate the maximum and minimum values.

Kaoline was mixed with *C. parvum* oocysts suspensions using two different methods. The $\log_{10}$ inactivation of *C. parvum* oocysts in excystation and animal infectivity that were subjected to UV irradiation in the presence of kaoline is shown in Figures 5 and 6. In experiments on mixtures of *C. parvum* oocysts and kaoline in which kaoline did not adhere to the oocyst wall, UV irradiation was

slightly more potent in *C. parvum* oocyst suspensions in water containing kaoline (1.99 log₁₀ inactivation in the excystation assay and 3.18 log₁₀ inactivation in the animal infectivity assay) than in oocyst suspensions in pure water. However, because the increase in potency was negligible, we did not study whether this increase resulted from scattering of the UV beam by kaoline particles. In experiments on mixtures of *C. parvum* oocysts and kaoline in which kaoline adhered to the oocyst wall, the level of UV inactivation of *C. parvum* oocysts tended to be reduced, as was seen with adhesion of humin. However, the magnitude of the log₁₀ inactivation with adhesion of kaoline was only approximately 10% less than that in pure water, irrespective of whether assessment was performed by excystation assay or by animal infectivity assay. These results suggest that the presence of adherent substances reduces the magnitude of the UV-induced log₁₀ inactivation of *C. parvum* oocysts by 20% or less, regardless of whether the substance is dispersed in water or partly adhered to the oocyst wall.

Figure 5. The log₁₀ inactivation of *C. parvum* oocysts in excystation upon UV irradiation in the presence of kaoline. Bars indicate the means from two independent experiments, and the lines indicate the maximum and minimum values.

Figure 6. The log₁₀ inactivation of *C. parvum* oocysts in animal infectivity upon UV irradiation in the presence of kaoline. Bars indicate the means from two independent experiments, and the lines indicate the maximum and minimum values.

Conclusions

The results of the present study can be summarized as follows:

- (1) Although the dose of UV delivered to oocysts decreases due to the influence of turbid substances, the degree of expected inactivation can be estimated when the dose of UV delivered to oocysts is known.
- (2) Humin and kaoline each reduced the magnitude of the UV-induced log₁₀ inactivation of *C. parvum* oocysts by 20% or less, regardless of whether the substance was dispersed in the water or partially adhered to the oocyst wall.

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