

## Comparison of UV Inactivation of Spores of Three *Encephalitozoon* Species with That of Spores of Two DNA Repair-Deficient *Bacillus subtilis* Biodosimetry Strains

Marilyn M. Marshall,<sup>1\*</sup> Samuel Hayes,<sup>2</sup> Jackie Moffett,<sup>1</sup> Charles R. Sterling,<sup>1</sup>  
and Wayne L. Nicholson<sup>1</sup>

Department of Veterinary Science and Microbiology, University of Arizona, Tucson, Arizona 85721,<sup>1</sup> and  
U.S. Environmental Protection Agency, Cincinnati, Ohio 45268<sup>2</sup>

Received 14 June 2002/Accepted 18 October 2002

**When exposed to 254-nm UV, spores of *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, and *Encephalitozoon hellem* exhibited 3.2-log reductions in viability at UV fluences of 60, 140, and 190 J/m<sup>2</sup>, respectively, and demonstrated UV inactivation kinetics similar to those observed for endospores of DNA repair-defective mutant *Bacillus subtilis* strains used as biodosimetry surrogates. The results indicate that spores of *Encephalitozoon* spp. are readily inactivated at low UV fluences and that spores of UV-sensitive *B. subtilis* strains can be useful surrogates in evaluating UV reactor performance.**

UV technologies for disinfection of municipal drinking water have been in use for several years in Europe (6), and interest in UV is rapidly gaining momentum as a choice for North American water treatment. In fact, field testing, installation, and actual operation of UV systems have already begun at a number of North American sites. UV disinfection of municipal drinking water has become increasingly attractive for the following reasons. First, it has been discovered recently that oocysts of protozoan intestinal parasites such as *Cryptosporidium parvum*, which are resistant to chlorination, are readily inactivated by relatively low doses of UV (2, 3). Second, UV treatment of water results in little or no formation of harmful disinfection by-products (5).

The microsporidian intestinal parasite *Encephalitozoon intestinalis* has recently also been placed on the first Contaminant Candidate List under the Safe Drinking Water Act (4), and two other microsporidian species, *Encephalitozoon cuniculi* and *Encephalitozoon hellem*, are known to be pathogenic for humans (15), but to date only *E. intestinalis* has been reported to be sensitive to UV radiation at a dose of 60 J/m<sup>2</sup> (7). However, the sensitivities of *E. cuniculi* and *E. hellem* to UV disinfection have not been evaluated.

United States Environmental Protection Agency (EPA) regulators are currently assessing UV disinfection as a key component of the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) (13). Under the LT2ESWTR, validation tests will be necessary to prove that any given UV reactor conforms to the standards of drinking water disinfection, which will be determined by the EPA. UV reactor validation testing is used to determine conditions that can be monitored to ensure delivery in routine operation of the proper UV fluence required for adequate water disinfection. Physical monitoring of applied irradiation by using electronic

UV sensors can be problematic and may not yield an accurate picture of the biologically effective UV fluence in large-scale municipal settings (for discussion, see reference 6). Therefore, to determine the biologically effective fluence delivered by a UV reactor, one must rely on more direct measures, such as biodosimetry, the inactivation of a nonpathogenic test organism whose UV dose-response characteristics have been precisely quantified (1, 6). *Escherichia coli* bacteriophage MS-2 and spores of *Bacillus subtilis* strain ATCC 6633 have been used extensively as UV biodosimetry reference organisms (biodosimeters). The 254-nm UV fluences required to cause a 4-log inactivation of the MS-2 and *B. subtilis* biodosimeters are approximately 800 and 600 J/m<sup>2</sup>, respectively (6, 14), which greatly exceed the 4-log inactivation fluence for *C. parvum* oocysts, which is only approximately 80 J/m<sup>2</sup> (2, 3). If information is required as to how closely a UV reactor system approaches the performance of an ideal system, reactor validation should include at least two biodosimeters of widely different UV sensitivities, with one exhibiting a UV sensitivity similar to or greater than that of the target pathogen and the other exhibiting greater UV resistance than the target pathogen (1).

The present study had two objectives. The first objective was to define the sensitivities of *E. intestinalis*, *E. cuniculi*, and *E. hellem* to 254-nm UV radiation. The second objective was to investigate the use of spores of *B. subtilis* DNA repair-deficient strains as biodosimeters that would exhibit inactivation kinetics similar to those of the microsporidian spores when irradiated with 254-nm UV under identical conditions. For comparative purposes, experiments were performed in parallel at the EPA facility in Cincinnati, Ohio (EPA), and at the University of Arizona, Tucson (UA).

Spores of *E. cuniculi* ATCC 50502, *E. hellem* ATCC 50451, and a duodenal isolate of *E. intestinalis*, ATCC 50603, were purified weekly from stock flasks, enumerated by hemocytometry (15), and stored in phosphate-buffered saline or sterile deionized water at 4°C (UA). *B. subtilis* strains WN333 (*trpC2 ΔsplAB::ermC1*) (10) and WN626 (*uvrB42 ΔsplAB::ermC1 amyE::tet*) (this study) were used for biodosimetry. *B. subtilis*

\* Corresponding author. Mailing address: Department of Veterinary Science and Microbiology, Building 90, Room 304, University of Arizona, Tucson, AZ 85721. Phone: (520) 621-4439. Fax: (520) 621-6366. E-mail: marshalm@u.arizona.edu.

TABLE 1. UV inactivation kinetics of *E. intestinalis*, *E. hellem*, and *E. cuniculi* spores<sup>a</sup>

| Spores                 | Site | UV fluence (J/m <sup>2</sup> ) | No. of infected/<br>no. inoculated (P) | Response<br>logit | Live<br>spores (D) | Log applied<br>dose (D <sub>0</sub> ) | Log change<br>(D/D <sub>0</sub> ) | Temp (°C) |
|------------------------|------|--------------------------------|--|-------------------|--------------------|---------------------------------------|-----------------------------------|-----------|
| <i>E. intestinalis</i> | UA   | 50                             | 141/5 (0.93)                           | 2.639057          | 75                 | 3.2                                   | -1.3                              | 25        |
|                        | UA   | 60                             | 1/10 (0.10)                            | -2.197225         | 1                  | 3.2                                   | -3.2                              | 25        |
|                        | EPA  | 60                             | 2/15 (0.13)                            | -1.8718           | 1                  | 3.2                                   | -3.2                              | 25        |
|                        | UA   | 80                             | 3/15 (0.20)                            | -1.386294         | 1                  | 3.2                                   | -3.2                              | 25        |
|                        | UA   | 90                             | 0/10 (0)                               |                   |                    | 3.2                                   | -3.2                              | 25        |
|                        | EPA  | 90                             | 0/10 (0)                               |                   |                    | 3.2                                   | -3.2                              | 25        |
|                        | UA   | 120                            | 0/5 (0)                                |                   |                    | 3.2                                   | -3.2                              | 25        |
|                        | UA   | 140                            | 0/5 (0)                                |                   |                    | 3.2                                   | -3.2                              | 25        |
|                        | UA   | 140                            | 0/5 (0)                                |                   |                    | 3.2                                   | -3.2                              | 5         |
| <i>E. hellem</i>       | UA   | 120                            | 8/10 (0.80)                            | 1.386294          | 55                 | 3.2                                   | -1.5                              | 25        |
|                        | UA   | 140                            | 5/10 (0.50)                            | 0                 | 16                 | 3.2                                   | -2.0                              | 25        |
|                        | UA   | 170                            | 2/10 (0.20)                            | -1.386294         | 4                  | 3.2                                   | -2.6                              | 25        |
|                        | UA   | 190                            | 0/10 (0)                               |                   |                    | 3.2                                   | -3.2                              | 25        |
| <i>E. cuniculi</i>     | UA   | 80                             | 6/10 (0.60)                            | 0.405465          | 29                 | 3.2                                   | -1.8                              | 25        |
|                        | UA   | 100                            | 4/10 (0.40)                            | -0.405465         | 8                  | 3.2                                   | -2.3                              | 25        |
|                        | UA   | 130                            | 2/10 (0.20)                            | -1.386294         | 2                  | 3.2                                   | -3.0                              | 25        |
|                        | UA   | 140                            | 0/5 (0)                                |                   |                    | 3.2                                   | -3.2                              | 25        |

<sup>a</sup> Calculations performed according to Korich et al. (8). See the text for details.

strains were routinely cultivated at 37°C on Luria-Bertani agar (9) containing the appropriate antibiotics at final concentrations of 1 µg of erythromycin/ml and 25 µg of lincomycin/ml (for WN333) and 10 µg of tetracycline/ml (for WN626). Spores were produced in Schaeffer's sporulation medium (12) and purified by lysozyme treatment and buffer washing as described previously (11) (UA). UV irradiation was performed with a standard low-pressure mercury vapor lamp (model UVGL-25; UVP, Inc., Upland, Calif.) (UA) or with a collimated beam apparatus containing two 15-W low-pressure UV lamps, model G15T8 (American UV Co., Lebanon, Ind.) as the light source and a UV reflector assembly, model XX-15S (UVP, Inc.) (EPA). Both devices emit predominantly 254-nm UV. UV fluences were measured by using either a UVX radiometer (UVP, Inc.) fitted with the appropriate calibrated probe for 254-nm UV (model UVX-25) (UA) or a model IL-1700 radiometer fitted with detector model SED240, an NS254 filter, and a wide-eye diffuser (International Light, Inc. Newburyport, Mass.) (EPA). Both UA and EPA radiometer units were calibrated by their respective manufacturers to a U.S. National Institutes of Standards and Technology standard. Furthermore, both instruments were directly compared to one another at UA by using the same UV source and were found to be in agreement. The UV irradiance distribution across the surface to be treated was predetermined by measuring the 254-nm irradiance at 0.5-cm intervals along the x-y axes of a 6-cm-diameter grid originating at the center of the UV beam. The average incident irradiance over the entire 6-cm-diameter petri dish was calculated by using UVCalc, a Microsoft Excel worksheet devised by James Bolton and kindly posted on the International UV Association website (<http://www.iuva.org>). *B. subtilis* and *Encephalitozoon* spores were diluted from stock suspensions into sterile deionized water to a final concentration of  $1 \times 10^7$  spores per 10 ml. The absorbances at 254 nm of the spore suspensions were measured in a UV spectrophotometer and entered into UVCalc to derive the exposure time needed for each final UV dose used. Spore suspensions were pipetted into an open 6-cm-diameter petri dish set atop a

rotating platform and exposed to the indicated UV doses. Serial 10-fold dilutions of *B. subtilis* spores were plated on Schaeffer's sporulation medium containing the appropriate antibiotics and incubated overnight at 37°C for viable colony counts. *B. subtilis* spore inactivation was calculated by using the formula  $\log_{10}$  inactivation =  $\log_{10}(N_0/N_t)$ , where  $N_0$  and  $N_t$  stand for the CFU of the suspension at the exposure times zero and  $t$ , respectively.

Logit dose-response relationships were developed for untreated fresh spores of the three *Encephalitozoon* species by titrating spore inocula by cell culture techniques as follows (8). The *E. intestinalis*, *E. cuniculi*, and *E. hellem* spores were inoculated onto RK-13-seeded 15-mm Thermanox coverslips of a 24-well microtiter plate and incubated for 7 days as previously described (16). Wells showing one or more infected cells were scored as positive, and those without infection were scored as negative. The proportion of infected wells (P), obtained by dividing the number of positive wells infected by the total number of wells inoculated, was used to calculate the response logit for each inoculum: response logit =  $\ln[P/(1 - P)]$ . Dose-response functions for each species were determined by linear regression analysis of the response logit versus the  $\log_{10}$  of each dose of spores producing a positive score (8). The resulting regression equations of the form response logit =  $m(\log_{10} \text{dose}) + b$  were used to estimate the number of viable spores remaining after treatment. This number was used to calculate the log change of viability as the  $\log_{10}$  of the estimated viable spores (D) divided by the total number of spores in each inoculum (D<sub>0</sub>) (i.e., log change + log [D/D<sub>0</sub>]) (8).

The results of 145 wells from the in vitro assay showed that the most sensitive spores of the three microsporidian species tested with low-pressure UV light were those of *E. intestinalis*, which exhibited 3.2-log inactivation at 60 J/m<sup>2</sup>. *E. cuniculi* and *E. hellem* spores showed 3.2-log inactivation at 140 J/m<sup>2</sup> and 190 J/m<sup>2</sup>, respectively (Table 1). Thus, in this study *E. intestinalis* spores exhibited a degree of UV sensitivity comparable to that of both *C. parvum* oocysts (2, 3) and *E. intestinalis* spores from a recently published study (7). Furthermore, spores of *E.*

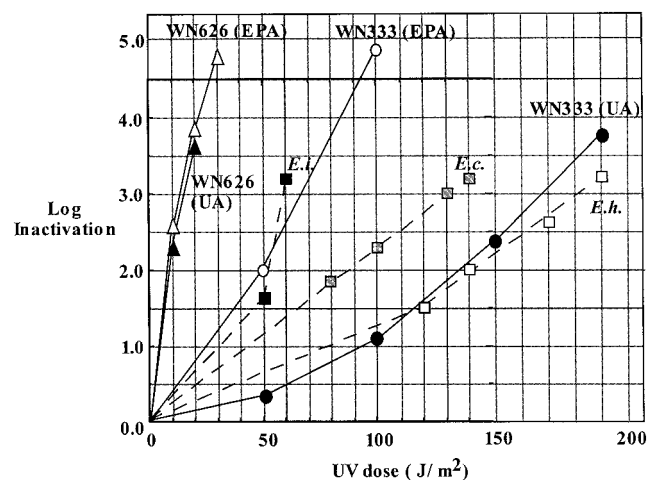


FIG. 1. Summary of spore inactivation by 254-nm UV. *B. subtilis* biosimetry strains WN333 (circles) and WN626 (triangles) at EPA (open symbols) or at UA (filled symbols). For comparison, the UV inactivation kinetics from Table 1 are plotted for spores of *E. intestinalis* (solid squares), *E. cuniculi* (hatched squares), and *E. hellem* (open squares). The data points shown for WN333 at UA are averages of three separate determinations, which varied by  $\pm 5\%$ .

*cuniculi* and *E. hellem* were observed to be approximately twice as resistant to UV as *E. intestinalis* spores. In all cases, a UV fluence of approximately 200 J/m<sup>2</sup> or higher would be predicted to inactivate each of the three pathogenic microsporidian species tested.

The UV inactivation kinetics of two *B. subtilis* biosimetry strains, WN333 and WN626, were determined in parallel at EPA and at UA. Spores of strain WN626 were observed to be extremely sensitive to UV, exhibiting between 3.5- and 4-log reduction at only 20 J/m<sup>2</sup> (Fig. 1). The data from the two laboratories concerning UV sensitivity of WN626 spores were in excellent agreement (Fig. 1). The UV sensitivity of spores of *B. subtilis* strain WN333 was found to vary by roughly a factor of 2 between the determinations at EPA versus those at UA (Fig. 1). The UV fluence required for 4-log inactivation of WN333 spores was found to be approximately 85 J/m<sup>2</sup> at EPA and 195 J/m<sup>2</sup> at UA (Fig. 1), which is likely due to differences in the enumeration techniques used at the two locations (membrane filters were used at EPA, and spread plates were used at UA). At both locations, however, WN333 spores exhibited UV inactivation kinetics similar to those of spores of the three *Encephalitozoon* species tested (Fig. 1).

The experiments described above were performed at room temperature (ca. 25°C). However, the water temperature in many municipal water treatment situations can be considerably lower, which we thought might affect the UV inactivation kinetics of microsporidian spores. In order to test this notion, spores of *E. intestinalis* were irradiated at UA to a final fluence of 140 J/m<sup>2</sup> at either 25°C or 5°C and exhibited >3.2-log inactivation at both temperatures (Table 1). Therefore, spores irradiated at 5°C were not more resistant to UV. In addition, the UV inactivation kinetics of *B. subtilis* WN333 and WN626 spores at UA were found to be the same at 25°C and 5°C (data not shown).

In conclusion, the data presented here indicate that spores of the microsporidian species *E. intestinalis*, *E. cuniculi*, and *E. hellem* exhibit UV inactivation kinetics similar to those of *C.*

*parvum* oocysts and that both microsporidian spores and oocysts would be inactivated by 254-nm UV at a fluence of 200 J/m<sup>2</sup>. Spores of *B. subtilis* biosimetry strains WN333 and WN626 closely mimic the *Encephalitozoon* spore (Fig. 1) and *C. parvum* oocyst (2, 3) dose-response curves and thus provide suitable surrogates for *Encephalitozoon* and *Cryptosporidium* spp. in UV reactor validation studies. *B. subtilis* spores have several advantages for use as a biosimetry surrogate. (i) They are nonpathogenic. (ii) They do not require eukaryotic cell culture or animal facilities. (iii) They afford testing results in  $\leq 24$  h (as opposed to 6 to 10 days required for the *Encephalitozoon* in vitro cell culture assay). (iv) They can be used in-house by any municipal water testing facility equipped to perform basic microbiology. The ease and speed of manipulation and the potential production capability make *B. subtilis* DNA repair-deficient spores strong potential candidates for use as biosimetry surrogates in a broad variety of UV testing and verification applications.

We thank Belinda Galeano for excellent technical assistance, Thomas Hargy for insightful comments and Dick Korich for critical review of the manuscript.

This work was supported by U.S. EPA Cooperative Agreement No. R-82883501-0.

#### REFERENCES

- Bolton, J. R., E. R. Blatchley III, A. Cabaj, W. Cairns, O. Hoyer, K. G. Linden, R. O. Rahn, M. Sages, R. Sommer, and E. Whitby. 2000. Terms and definitions in ultraviolet disinfection. Proceedings of Disinfection 2000, New Orleans, La. Water Environment Federation, Alexandria, Va. [On line.]
- Clancy, J. L., T. M. Hargy, M. M. Marshall, and J. Dyksen. 1998. Inactivation of oocysts of *Cryptosporidium parvum* in water using ultraviolet light. J. Am. Water Works Assoc. 90:92-102.
- Clancy, J. L., Z. Bukhari, T. M. Hargy, J. R. Bolton, B. W. Dussert, and M. M. Marshall. 2000. Using UV to inactivate *Cryptosporidium*. J. Am. Water Works Assoc. 92:97-104.
- Federal Register. 1998. Announcement of the Drinking Water Contaminant Candidate List. Fed. Regist. 63:10273-10287.
- Haider, T., R. Sommer, S. Knasmüller, P. Eckl, W. Pribil, A. Cabaj, and M. Kundi. 2002. Genotoxic response of Austrian groundwater samples treated under standardized UV (254 nm): disinfection conditions in a combination of three different bioassays. Water Res. 36:25-32.
- Hoyer, O. 2000. The status of UV technology in Europe. Int. UV Assoc. News 2:22-27.
- Huffman, D. E., A. Gennaccaro, J. B. Rose, and B. W. Dussert. 2002. Low- and medium-pressure UV inactivation of microsporidia *Encephalitozoon intestinalis*. Water Res. 36:3161-3164.
- Korich, D. G., M. M. Marshall, Z. Bukhari, H. V. Smith, J. O'Grady, C. R. Fricker, J. Rosen, and J. L. Clancy. 2000. Inter-laboratory comparison of the CD-1 neonatal mouse dose response model for *Cryptosporidium parvum* oocysts. J. Eukaryot. Microbiol. 47:294-298.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Nicholson, W. L., L. Chooback, and P. Fajardo-Cavazos. 1997. Analysis of spore photoproduct lyase operon (*splAB*) function using targeted deletion-insertion mutations spanning the *Bacillus subtilis* operons *ptsHI* and *splAB*. Mol. Gen. Genet. 255:587-594.
- Nicholson, W. L., and P. Setlow. 1990. Sporulation, germination, and outgrowth, p. 391-450. In C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Sussex, England.
- Schaeffer, P., J. Millet, and J.-P. Aubert. 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA 54:704-711.
- Scharfenaker, M. A. 2002. Draft LT2ESWTR out of the box. J. Am. Water Works Assoc. 94:24-37.
- Sommer, R., W. Pribil, S. Appelt, P. Gehringer, J. Eschweiler, H. Leth, A. Cabaj, and T. Haider. 2001. Inactivation of bacteriophages in water by means of non-ionizing (UV-253.7 nm) and ionizing (gamma) radiation: a comparative approach. Water Res. 35:3109-3116.
- Wittner, M., and L. M. Weiss. 1999. The microsporidia and microsporidiosis. American Society for Microbiology, Washington, D.C.
- Wolk, D. M., C. H. Johnson, E. W. Rice, M. M. Marshall, K. F. Grahn, C. B. Plummer, and C. R. Sterling. 2000. A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. *Septata intestinalis*. Appl. Environ. Microbiol. 66:1266-1273.