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Chlorine Inactivation of Spores of *Encephalitozoon* spp.

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This report is an extension of a preliminary investigation on the use of chlorine to inactivate spores of *Encephalitozoon intestinalis* and to investigate the effect of chlorine on two other species, *E cuniculi* and *E. hellem*, associated with human infection. The 50% tissue culture infective doses of these three species were also determined. On the basis of the results obtained, it appears that chlorination of water is an effective means of controlling spores of these organisms in the aquatic environment.

The phylum *Microspora* is composed of a diverse group of protistan parasites that infect a wide range of vertebrate and invertebrate hosts. Certain species of these obligate intracellular parasites are etiological agents of microsporidiosis in humans. While immunocompetent individuals may become infected, microsporidiosis is a particularly devastating disease in immunosuppressed individuals, especially those infected with the human immunodeficiency virus. The infective, spore stage of these organisms is released into the environment from infected hosts. By using molecular procedures, several studies have detected these organisms in water (3, 6). Recently, a report on a retrospective study of an outbreak of intestinal microsporidiosis implicated water as the probable vehicle of transmission (2). Interest in the possible role of these parasites as waterborne pathogens has prompted research into the efficacy of chlorination for controlling these organisms in water. This report is an extension of a preliminary investigation on the use of chlorine to inactivate spores of *Encephalitozoon intestinalis* (7) and to investigate the effect of chlorine on two other species, *E. cuniculi* and *E. hellem*, associated with human infection. The 50% tissue culture infective doses ($TCID_{50}$) of these three species were also determined.

E. cuniculi (ATCC 50502), *E. hellem* (ATCC 50451), and *E. intestinalis* (ATCC 50603) were propagated in monolayers of RK-13 (ATCC CCL-37) rabbit kidney cells incubated at 35°C in a humid atmosphere of 5% CO_2 as previously described (7). The spores were harvested by removing the culture medium from the cell monolayers and concentrated by centrifugation at 1,000 × *g* for 10 min. The supernatant was removed by aspiration, and the spore pellet was further purified by differential density gradient centrifugation by using an isopycnic Percoll (Amersham Pharmacia Biotech, Piscataway, N.J.) gradient and a final wash step using reagent grade water (7). The concentration of spores was determined by counting with a hemacytometer, and the stock preparations were stored at 4°C in reagent grade water. Spore preparations were used within 1 week of harvesting. Spores from individual species were used in the inactivation experiments.

$TCID_{50}$s were determined by using a logit dose-response model (5) to describe the dose-response relationship between the tissue culture wells and the *Encephalitozoon* sp. spores. Briefly, the response logit (RL) value was calculated for each inoculum of spores as the natural logarithm (ln) of the proportion of infected tissue culture wells (P) divided by 1 minus the proportion of inoculated wells $\{RL = \ln[P/(1 - P)]\}$. The RL values were treated as dependent variables (y) for linear regression analysis with the logarithm ($\log_{10}$) of the number of spores in each dose serving as the independent variable (x). Regression analysis was then used to perform a least-squares regression to calculate the regression model and parameters. The logit models were used to calculate the $TCID_{50}$ for each species and to determine the number of infective spores remaining in each inoculum after disinfectant treatment.

Inactivation experiments were conducted at 23 ± 2°C in chlorine demand-free (CDF) 0.05 M potassium dihydrogen phosphate buffer adjusted to pH 7.0 as previously reported (4). The reaction vessels used were 400-ml beakers containing 90 ml of CDF buffer. Reagent grade sodium hypochlorite was added to each beaker to achieve the desired level of chlorine prior to the addition of spores. An inoculum (10 ml) containing 10^7 spores was added to each reaction vessel at time zero. During the course of the experiments, the reaction vessel contents were continuously mixed with a magnetic stirring device. Ten-milliliter samples were removed from the reaction vessels at the desired exposure times, and the chlorine was immediately neutralized by the addition of 0.1 ml of 10% (wt/vol) sodium thiosulfate. Chlorine levels were determined initially and at each exposure time by the *N*,*N*-dimethyl-*p*-phenylenediamine colorimetric method (1). Vessels containing CDF buffer without chlorine served as controls for the unexposed spores and were treated in the same manner as the chlorine-exposed samples. Addition of sodium thiosulfate to the control vessel did not have a deleterious effect on the spores. The logit dose-response regression model (5) was used to determine the number of viable spores after chlorine treatment. The $\log_{10}$ reduction (LR) was determined by subtracting the number ($\log_{10}$) of viable spores remaining after treatment (N) from the

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TABLE 1. Infectivity of spores of *Encephalitozoon* spp. inoculated on monolayers of rabbit kidney cells (RK-13)

Species and no. of spores inoculated	No. of monolayers inoculated	No. positive	% Infected	Calculated $TCID_{50}$[a]
E. cuniculi				
10	15	6	60	
25	10	3	30	
100	15	11	73	15
E. hellem				
50	10	4	40	
100	15	9	60	68
E. intestinalis				
10	25	7	28	
100	45	29	64	27

[a] Number of spores.

total number (log_{10}) of spores in the initial inoculum (N_0) (LR = $log_{10}N - log_{10}N_0$).

The $TCID_{50}$ shown in Table 1 were determined by using the mean number of infected tissue culture wells at the various dilutions. Since the logit expressions for 0 and 100% infectivity are undefined, they were not used in the calculations. The calculated $TCID_{50}$ for *E. cuniculi* was 15 spores; for *E. hellem*, it was 68 spores, and for *E. intestinalis*, it was 27 spores. The results of the chlorine inactivation experiments are shown in Table 2. The chlorine level at the beginning of each experiment was 2.5 ± 0.2 mg $liter^{-1}$. The spore preparations exerted an initial rapid chlorine demand, and the residual chlorine level at the end of each exposure time was 2.0 ± 0.2 mg $liter^{-1}$. *E. hellem* showed a greater degree of resistance than the other two species at the shorter exposure times. However, all three species were inactivated by 4 orders of magnitude or more after an exposure time of 6 to 8 min, equating to a simple *Ct* value (*Ct* = disinfectant concentration [milligrams per liter] × exposure time [minutes]) of 16 mg-min $liter^{-1}$.

The $TCID_{50}$ for *E. cuniculi* and *E. hellem* reported here are the first published values for these species. The currently reported $TCID_{50}$ of 27 spores for *E. intestinalis* is considerably lower than the previously reported value of 915 (7). The same procedure and reagents were used in both studies, with the only variable being the serum, which was obtained from a different vendor for the present study. This finding underscores the variability that can occur in the determination of $TCID_{50}$ and the necessity of including individual control cultures for each organism when conducting inactivation experiments. These results confirm the previous report of Wolk et al. (7) showing that spores of *E. intestinalis* are sensitive to chlorination and further indicate that other species of *Encephalitozoon* are also capable of being inactivated by chlorine. The level of chlorination (*Ct* = 16) required to inactivate spores of *Encephalitozoon* spp. is readily attainable by most water utilities in the United States (4). On the basis of these results, it appears that chlorination of water is an effective means of controlling spores of these organisms in the aquatic environment.

TABLE 2. Chlorine inactivation of spores of *Encephalitozoon* spp.[a]

Species and exposure time (min)	No. of spores inoculated	No. of monolayers inoculated	% Infected	Calculated log_{10} inactivation
E. cuniculi				
4	10^4	10	50	3.32
5	10^4	10	40	3.46
6	10^4	10	20	3.80
8	10^4	10	0	≥4.00
E. hellem				
4	10^3	10	90	0.70
5	10^3	10	60	1.10
6	10^4	10	10	2.67
8	10^4	10	0	≥4.00
E. intestinalis				
4	10^3	10	30	1.73
5	10^3	10	20	1.84
6	10^4	5	0	≥4.00

[a] In CDF chlorinated buffer, pH 7, 23 ± 2°C, 2.0 mg of free chlorine per liter.

REFERENCES

1. **American Public Health Association.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
2. **Cotte, L., M. Rabodonirina, F. Chapuis, F. Bailly, F. Bissuel, C. Raynal, P. Gelas, F. Persat, M. Piens, and C. Trepo.** 1999. Waterborne outbreak of intestinal microsporidiosis in persons with and without human immunodeficiency virus infection. J. Infect. Dis. **180:**2003–2008.
3. **Dowd, S. E., C. P. Gerba, and I. L. Pepper.** 1998. Confirmation of the human-pathogenic microsporidia *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Vittaforma cornea* in water. Appl. Environ. Microbiol. **64:** 3332–3335.
4. **Johnson, C. H., E. W. Rice, and D. J. Reasoner.** 1997. Inactivation of *Helicobacter pylori* by chlorination. Appl. Environ. Microbiol. **63:**4969–4970.
5. **Korich, D. G., M. M. Marshall, H. V. Smith, J. O'Grady, Z. Bukhari, C. R. Fricker, J. P. Rosen, and J. L. Clancy.** 2000. Inter-laboratory comparison of the CD-1 neonatal logistic dose-response model for *Cryptosporidium parvum* oocysts. J. Eukaryot. Microbiol. **47:**294–298.
6. **Sparfel, J. M., C. Sarfati, O. Liguory, B. Caroff, N. Dumoutier, B. Gueglio, E. Billaud, F. Raffi, J. Molina, M. Miegeville, and F. Derouin.** 1997. Detection of microsporidia and identification of *Enterocytozoon bieneusi* in surface water by filtration followed by specific PCR. J. Microbiol. **44:**78S.
7. **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.