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Dose Response of *Cryptosporidium parvum* in Outbred Neonatal CD-1 Mice

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***Cryptosporidium parvum* infectivity in a neonatal CD-1 mouse model was used to determine the dose needed to infect 50% of the population. The 50% infective dose was estimated to be 79 oocysts. It was observed that a mean oral inoculum of 23 oocysts produced infection in 2 of 25 neonatal mice 7 days postinoculation. All animals became infected when the mean oral dose exceeded 310 oocysts per animal. The dose response of *C. parvum* was modeled with a logit dose-response model suitable for use in water disinfection studies.**

Cryptosporidium parvum, an enteric parasite of vertebrates, was first reported to be a human pathogen in 1976 (19). While immunocompetent adults clear the infection 3 to 4 weeks after exposure to oocysts, immunocompromised individuals are unable to eliminate the parasites from the small intestine and suffer from chronic cryptosporidiosis (23). The recognition of *C. parvum* as a threat to immunocompromised individuals, such as AIDS patients, and the fact that the parasite was identified as the etiologic agent of several waterborne outbreaks of enteric illness have made the control of *C. parvum* a priority with public health officials and the water supply industry (5, 11). *C. parvum* has been reported to be resistant to many common disinfectants, including chlorine (3, 14). Currently, there is no effective chemotherapy available for treatment of cryptosporidiosis (16).

There is a need for an animal model for studying the infectivity of *Cryptosporidium* oocysts. There are applications for such a model in the water treatment industry and in pharmaceutical research because of the requirement for measuring the infection-blocking capability of water disinfection processes or the efficacy of an antimicrobial agent. The neonatal mouse host for *C. parvum* was developed after attempts to infect other immunocompetent rodents failed to produce symptomatic or reproducible infections (7). The 50% infective dose (ID_{50}) in neonatal Swiss-Webster mice has been estimated to be between 100 and 500 oocysts (7). Others reported the ID_{50} to be 60 oocysts in neonatal BALB/c mice, although no data were provided (14). Little information is available on the infective dose of *C. parvum* in primates, although one study reported that inoculation with either 10 or 2×10^5 oocysts by means of a nasogastric tube resulted in clinical enteritis (17). The size of the inoculum had no apparent effect on the severity or duration of disease.

The purpose of this study was to develop the dose-response characteristics for *C. parvum* in neonatal CD-1 mice. The objectives include determining the ID_{50} for this mouse strain and the minimum infectious dose. The data from this study can be used as a guide for researchers attempting to quantify the inactivation of *C. parvum* oocysts when using agents such as chemical disinfectants in water treatment.

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MATERIALS AND METHODS

Production and purification of *C. parvum* oocysts. One of us obtained the original strain of *C. parvum* oocysts used in this study from Charles R. Sterling (Department of Veterinary Science, University of Arizona, Tucson), who originally obtained this isolate from Harley Moon (National Animal Disease Center, Ames, Iowa). The oocysts used in this study were produced in male neonatal holstein calves (*Bos tauris*) after a modification of the methods described by Musial et al. (18). A calf that was obtained at birth was given up to 2 liters of colostrum from a bottle. Within 12 h the animal was dosed from a bottle containing 2×10^8 *C. parvum* oocysts suspended in 2 liters of milk replacer. The calf was maintained on a diet of 1 part milk replacer and 1 part electrolyte solution during fecal collection.

At the onset of scouring, the feces were collected in tap water and sequentially passed through 10-, 20-, 60-, 100-, 200-, and 400-mesh sieves (ATM Test Sieves, Inc., Milwaukee, Wis.) by agitating and washing the sieves with 0.01% (vol/vol) aqueous Tween 20. Concentration of the particulates from the sieved feces was by centrifugation at 1,100 × *g* for 10 min. Purification of the oocysts from the particulates was done on a step gradient of Sheather's sucrose (500 g of sucrose, 320 ml of distilled deionized water, 9 ml of phenol). In a 50-ml conical centrifuge tube, the equivalent of 1 ml of particulates was diluted to 25 ml with 0.01% Tween 20 and suspended by vortexing. The fecal slurry was underlaid with 15 ml of 1:4 Sheather's sucrose (200 ml of Sheather's sucrose, 800 ml of phosphate-buffered saline, 9 ml of Tween 80; specific gravity, 1.064). The 1:4 Sheather's sucrose band in turn was underlaid with 10 ml of 1:2 Sheather's sucrose (300 ml of Sheather's sucrose, 600 ml of phosphate-buffered saline, 9 ml of Tween 80; specific gravity, 1.103). The tube was spun in a swinging-bucket rotor at 1,100 × *g* for 20 min with gradual acceleration and deceleration and without braking. The interface between the two Sheather's sucrose bands was collected, diluted five times with 0.01% Tween 20, and centrifuged at 1,100 × *g* for 10 min. The resultant oocysts were washed two more times in 0.01% Tween 20 and centrifuged at 1,100 × *g* for 10 min. Normally, phase microscopic examination of the oocyst suspension at this point indicated that the suspension was still contaminated with fecal debris. Consequently, secondary and tertiary Sheather's step gradients were done until the *C. parvum* oocyst suspension was purified to near homogeneity. After

the final wash step, the oocysts were suspended in 0.01% Tween 20 containing 100 U of penicillin, 100 μg of streptomycin, and 100 μg of gentamicin per ml and stored at 4°C. The oocyst concentration of the suspension was determined by counting 0.01% Tween 20 dilutions with a hemocytometer. The typical concentration of oocysts in the stock suspension was 1.5×10^8 to 2.5×10^8 per ml. The *C. parvum* oocysts were never exposed to 2.5% potassium dichromate as is commonly done (4).

Infectivity. The neonatal mouse model described by Ernest et al. (7) was used to evaluate the infectivity of *C. parvum*. Breeding pairs of CD-1 outbred mice were obtained from the Charles River Breeding Laboratories (St. Constant, Quebec, Canada). The animals were given food and water ad libitum and were housed in cages with covers fitted with a 0.22-μm-pore-size filter in a specific-pathogen-free (P-2 level) animal facility.

Oocyst doses were prepared from the stock suspension of oocysts by serial dilution to obtain dosages between 18 and 400 oocysts per mouse. The oral dose given to the mice was determined from the mean of four hemocytometer counts of the stock suspension and the dilution factor. The dose was not corrected for in vitro excystation rates. The Fisher D^2 statistic was used for quality control of the replicate counts (10). If the variance of the counts was within the amount expected from chance alone ($P \leq 0.05$), the mean count was used. Otherwise, the oocyst suspension was resampled and recounted.

The mice was inoculated orally 4 days after birth, using a pipette with known numbers of oocysts suspended in 5 to 10 μl of Milli-Q (Millipore Corp.) water. The infectivity of the oocysts was determined 7 days after infection. The mice were killed by cervical dislocation, and the lower half of the small intestine, the cecum, and the colon were removed and placed in 10 ml of Milli-Q water. The intestines were homogenized for 10 s in a Sorvall Omni-Mixer (Du Pont Co.) and washed three times in Milli-Q water containing 0.01% Tween 20 at $2,000 \times g$ for 15 min. After centrifugation, the supernatant was discarded, 10 ml of Sheather's sugar solution was added to the pellet, and the solution was centrifuged at $1,000 \times g$ for 10 min. A few drops from the surface of the suspension were removed and examined with differential interference contrast microscopy at ×400. Mice were scored either positive or negative for oocysts after examination of the slide.

In vitro excystation was used as a quality control procedure to monitor changes in the oocyst preparation over the duration of the experiments. The in vitro excystation method of Woodmansee (24) was used. No statistically significant difference ($P \leq 0.05$) was found between the method used in this study and an alternate method (14). Oocysts that had an excystation rate below 60% were not used in experiments. Prior to excystation, 1.5% of the stock oocyst preparation was shells.

RESULTS

Table 1 summarizes the oral dose per animal, the number of animals receiving the dose, and the number of animals that were infected as determined by examination of the small intestine. The standard error of the mean dose, the age of the oocysts, and the dilution factors are also given in Table 1. A logit dose-response model for *C. parvum* in neonatal mice was developed with data from this study (9). The logit response as a function of *C. parvum* oocyst dose is shown in Fig. 1 with 90% confidence limits on the response. The parameters for the logit model were estimated by using the method of least squares. The resulting model was the following: response logit = $-6.738 + 3.547 \log_{10}$ (inoculum) (equation 1), where the response logit is $\ln[P/(1 - P)]$ and P is the proportion of animals infected for the specified inoculum. Alternate models have been proposed for dose-response data (2, 8, 12, 13, 15). However, it has been suggested that the log-normal model similar to the logit model used in this study gave similar results when compared with more sophisticated statistical models (12).

With equation 1, the ID_{50} (logit = 0) for oocysts in neonatal CD-1 mice was found to be 79 oocysts per animal. At the minimum dose level used in this study (dose group 1), it was observed that 25 oocysts caused an infection in 2 of the 25 mice after 7 days. The two positive mice were from the same litter. In dose group 10, it was observed that 400 oocysts caused infection in 11 of 11 inoculated mice.

DISCUSSION

At the lowest mean dose of 23 oocysts per animal, infection was found in 2 of 25 animals. Interestingly, the two positive mice were both from the same litter. If one less litter had been used, it is quite possible that no animals would have been scored positive at the lowest inoculum. It is possible that the lowest dose level that was used in this study was near the threshold of the minimum infective dose for the neonatal mouse model. At a mean oral dose of 400 oocysts per animal, 100% of the animals responded with an infection. There was an apparent inconsistency in the dose response for data obtained for doses of 81 to 88 oocysts. No explanation of this anomaly could be found.

An earlier study used neonatal Swiss-Webster mice and examined three dose levels: 107 ± 5 oocysts, 537 ± 8 oocysts, and 1,096 ± 10 oocysts per animal (7). There were 32 animals exposed at each dose level. The results of that study are summarized in Fig. 2 alone with the results from this study. The dose-response model developed from the data of Ernest et al. (7) is as follows: response logit = $-5.996 + 2.576 \log_{10}$(inoculum) (equation 2). The ID_{50} calculated from this model is 210 oocysts per animal. The estimated ID_{50} from this study is 79. In the study of Ernest et al. (7), 22% of the animals exposed to 100 cysts became positive, whereas 39% of the subjects became positive in the present study at a similar dose. However, examining Table 1 reveals that a logit response of about 0 (corresponding to the ID_{50}) occurs at a little less than 140 oocysts per animal. Given the inherent variability in an animal model system such as this one, and given that prior knowledge of the infective proportion of the oocyst suspension is not available, the dose-response model of Ernest et al. (7) is in reasonable agreement with that reported in this study.

There are several sources of variation in the animal model system, including the strain of mouse used, the age of the mouse, the infectivity of the oocysts, and the strain of *C. parvum*. Enriquez and Sterling (6) found that of 19 different strains of mice, only the adult beige mouse (C57BL/6J-bg) and the BALB/c neonates were susceptible to *C. parvum* after inoculation with 10^6 oocysts. CD-1 mice were not evaluated. Korich et al. (14) reported an ID_{50} of 60 oocysts for neonatal BALB/c mice, but the authors failed to provide the raw data supporting the calculation. The data from the present study show that the CD-1 strain is susceptible to *C. parvum* and is an economical choice for experiments. However, it was found that while the progeny of most CD-1 females and sires in this study were highly susceptible to

TABLE 1. Summary of *C. parvum* oocyst doses and responses in 4-day-old CD-1 mice

Dose group	Trial	Age of oocysts (days)	Mean initial count (oocysts per ml)	Dilution factor	Dose (oocysts per mouse)	No. of animals	No. of animals infected	Mean dose (oocysts per mouse)	SEM dose	Response logit
1	17	97	2.0E + 08[a]	1.1E + 07	18	9	0	23	2.2	−2.44
	19	112	3.9E + 08	1.6E + 07	25	10	2			
	32	155	1.6E + 08	6.3E + 06	25	6	0			
2	26	140	1.6E + 08	4.0E + 06	40	6	1	41	0.9	−1.10
	34	178	1.6E + 08	4.0E + 06	40	5	2			
	30	149	1.6E + 08	3.8E + 06	43	5	1			
3	1	51	1.4E + 07	2.9E + 05	50	8	0	53	1.1	−1.25
	15	95	2.0E + 08	4.0E + 06	50	6	1			
	36	180	1.6E + 08	2.9E + 06	53	4	1			
	42	141	3.6E + 08	6.6E + 06	55	5	2			
	43	151	3.6E + 08	6.5E + 06	55	4	2			
4	39	112	3.7E + 08	5.7E + 06	64	4	1	72	2.6	0.51
	20	112	3.9E + 08	5.3E + 06	73	7	5			
	16	95	2.0E + 08	2.7E + 06	75	8	5			
	23	133	1.1E + 08	1.4E + 06	75	5	4			
5	38	105	3.3E + 08	4.1E + 06	81	8	6	84	1.7	2.44
	41	130	3.7E + 08	4.5E + 06	81	4	4			
	31	149	1.6E + 08	1.9E + 06	85	5	5			
	5	76	5.2E + 07	5.9E + 05	88	8	8			
6	2	51	1.4E + 07	1.4E + 05	100	8	1	100	1.9	−0.43
	8	80	5.1E + 07	5.1E + 05	100	8	2			
	13	91	4.9E + 07	4.9E + 05	100	8	1			
	37	180	1.6E + 08	1.5E + 06	110	4	4			
	28	141	1.6E + 08	1.5E + 06	110	5	5			
7	24	133	1.1E + 08	8.5E + 05	120	5	5	140	4.8	0.25
	40	112	3.7E + 08	2.8E + 06	130	4	4			
	35	178	1.6E + 08	1.2E + 06	130	7	7			
	3	51	1.4E + 07	9.6E + 04	150	8	3			
	14	91	4.9E + 07	3.2E + 05	150	9	1			
	9	80	5.1E + 07	3.3E + 05	150	6	1			
	6	76	5.2E + 07	3.3E + 05	160	9	6			
8	18	97	2.0E + 08	1.1E + 06	180	6	4	210	8.4	1.39
	7	76	5.2E + 07	2.6E + 05	200	6	6			
	4	51	1.4E + 07	7.2E + 04	200	7	4			
	10	80	5.1E + 07	2.5E + 05	200	10	5			
	12	83	5.1E + 07	2.5E + 05	200	7	7			
	21	114	1.7E + 08	7.7E + 05	220	8	8			
	33	155	1.6E + 08	6.3E + 05	250	6	6			
9	22	114	1.7E + 08	5.8E + 05	300	8	8	310	8.5	1.85
	11	80	5.1E + 07	1.7E + 05	310	9	6			
	29	141	1.6E + 08	4.9E + 05	330	5	5			
10	25	133	1.1E + 08	2.7E + 05	400	5	5	400	0.0	∞
	27	140	1.6E + 08	4.0E + 05	400	6	6			

[a] 2.0E + 08 = 2.0×10^8.

infection with greater than 150 oocysts, the progeny of others were not. A range of susceptibilities to parasitic infections is common among animals (1, 22). Although the evidence for differential susceptibility of humans to *Cryptosporidium* oocysts is not available at present, studies (including the present one) suggest a possible influence of host genetics on susceptibility to *Cryptosporidium* protozoa (6, 21). The immune status of the female mice can also have an effect on the offspring, and prior exposure of some dams to *Cryptosporidium* protozoa may have an effect on the resistance of the offspring (under investigation).

Novak and Sterling (20) reported that the age of the neonatal mouse affects the parasite load in the intestine. Mice inoculated at 14 days of age and examined 10 days later had fewer intestinal parasites than those inoculated at 4 days of age and examined 5 days later. Susceptibility of the mice to *C. parvum* decreased with age, and at 10 days old, some of the mice were already resistant to infection. The dose-

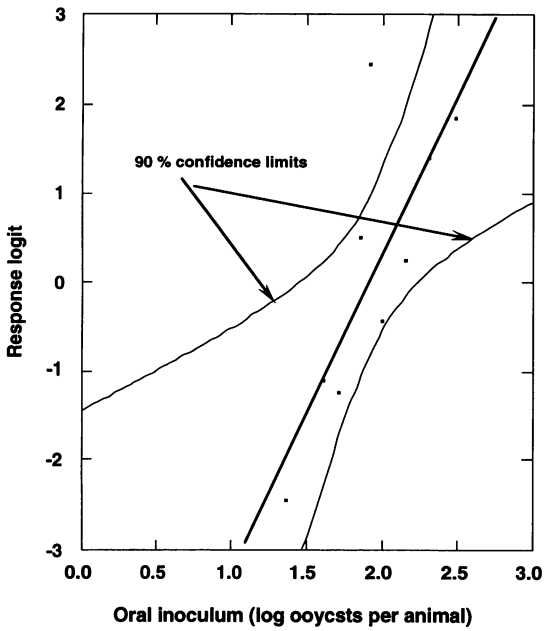

FIG. 1. 90% confidence limits for the response logit of *C. parvum* oocysts inoculated orally into neonatal CD-1 mice.

response data in this study were generated only with neonates that were 4 days old.

The infectious dose of *C. parvum* in humans is not presently known. Miller et al. (17) reported that inoculation of macaque monkeys (*Macaca nemestrina*) with 10 and 2 × 10^5 oocysts each resulted in infection. Only two animals were used at each dose level. The authors also suggested that the ID_{90} for infant macaques was 10 to 50 oocysts on the basis of their experience in a primate care facility.

The data obtained in this study are valuable in that they are consistent with previous work with mouse model systems for *C. parvum*. In addition, the definition of the model permits its use in chemical disinfection studies of waterborne *C. parvum* oocysts. However, in the absence of data regarding the dose response of *Cryptosporidium* oocysts in humans, the mouse model has no facility in predicting the risks for humans exposed to *Cryptosporidium* protozoa.

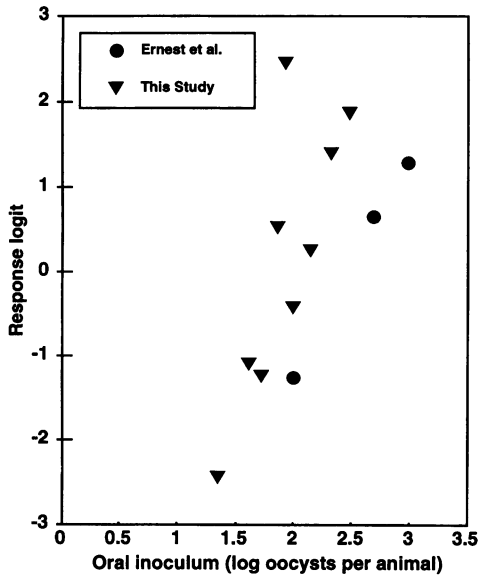

FIG. 2. Comparison of the results of this study and previously reported data on the dose response of *C. parvum* oocysts in neonatal mice.

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