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Neonatal-Mouse Infectivity of Intact *Cryptosporidium parvum* Oocysts Isolated after Optimized In Vitro Excystation

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We reexamined the finding of Neumann et al. (10) that intact *Cryptosporidium parvum* oocysts obtained after in vitro excystation were infectious for neonatal CD-1 mice. We used both established excystation protocols and our own protocol that maximized excystation (2). Although intact oocysts isolated after any of three protocols were infectious for neonatal CD-1 mice, the infectivity of intact oocysts isolated with our optimized excystation protocol was significantly lower than the infectivity of intact oocysts isolated after established protocols or from fresh oocysts. Excystation should not be considered a valid measure of *C. parvum* viability, given that it is biologically implausible for oocysts to be nonviable and yet infectious.

The use of excystation as an indicator of *Cryptosporidium parvum* oocyst viability has come under criticism, given the observation that intact oocysts isolated after in vitro excystation were infectious for CD-1 neonatal mice (10). The presumption is that if an oocyst cannot excyst, it will be incapable of the physical and biochemical steps involved in initiating asexual multiplication (6, 7). Using established in vitro procedures, previous studies have found a wide discrepancy in the proportion of oocysts that excyst, ranging from 50 to 95% depending on which technique was used to stimulate excystation, such as pretreatment of oocysts with acid (2, 4, 5, 10, 12). Such a wide discrepancy in excystation rates for the same batch of oocysts indicates that some excystation protocols fail to fully stimulate oocysts to excyst, resulting in excessive amounts of intact oocysts following the procedure. This result not only leads to underestimating the proportion of oocysts that are presumably viable but also biases experiments that use intact oocysts isolated after in vitro excystation.

Our concern with the conclusion that intact oocysts isolated after in vitro excystation are infectious for CD-1 neonatal mice (10) is that the process of excystation appeared not to be optimized, resulting in substantial numbers of excystable (i.e., viable) oocysts failing to excyst, thereby explaining the infectious potential of such oocysts for neonatal mice. Our goal for this project was to reexamine the finding that intact *C. parvum* oocysts obtained after in vitro excystation were infectious for neonatal CD-1 mice, using a protocol that maximized the proportion of oocysts that excysted (2), resulting in a more valid population of intact, presumably nonviable oocysts for in vivo experimentation.

***C. parvum* oocysts.** Feces were collected from naturally infected calves at 9 to 21 days of age from three local commercial dairies in Tulare, Calif., which served as the source of wild-type *C. parvum* oocysts for these experiments. These oocysts were previously classified as bovine genotype A, using the genotyping scheme described by Xiao et al. (13). After an acid fast-staining procedure was used to detect oocysts (9), samples having more than 25 oocysts per microscopic field (400×) of fecal smears were washed through a series of 40-, 100-, 200-, and 270-mesh sieves with Tween water (0.2% Tween 20 in deionized water [vol/vol]). The resulting suspension was centrifuged at 1,500 × *g* for 20 min in a 250-ml centrifuge tube, the supernatant was discarded, and the pellet was diluted in Tween water. Discontinuous sucrose gradients were used to purify *C. parvum* oocysts from fecal suspensions (1). Purified oocysts were stored in *Cryptosporidium* storage solution which contained 0.001% amphotericin, 0.006% penicillin G, 0.01% streptomycin sulfate, and 0.01% Tween 20. Using a phase contrast hemacytometer (Bright-Line; Hausser Scientific, Horsham, Pa.), concentrations of purified oocysts were determined as the arithmetic mean of eight independent counts. Oocysts were used within 2 to 7 days of fecal collection.

Protocol I. We attempted to replicate the protocol described by Black et al. (3). Two times excystation medium was made by dissolving 150 mg of sodium taurocholate (Sigma, St. Louis, Mo.) and 50 mg of trypsin (Sigma) in 5 ml of phosphate-buffered saline (PBS, pH 7.4; Sigma). Equal volumes of 2×10^7 oocysts/ml of suspension and 2× excystation medium were mixed on a vortex. The oocyst suspension was then incubated at 37°C for 3 h and then kept at room temperature for 30 min. The parasite suspension was centrifuged at 10,000 × *g* for 10 min, the supernatant was removed, and the excystation mixture was resuspended in PBS.

Protocol II. We attempted to replicate the protocol described by Rennecker et al. (11). The excystation medium consisted of 1.5% (wt/vol) taurocholate and 0.5% (wt/vol) trypsin dissolved in Hanks balanced salt solution (Sigma). One hundred microliters of an oocyst suspension (10^8 oocysts/ml) was added to 1 ml of the excystation medium, mixed on a vortex, and incubated at 37°C for 3 h. The oocyst suspension was centrifuged at 1,120 × *g*, the supernatant was removed, and the excystation mixture was resuspended in PBS.

Protocol III. Our procedure for protocol III was a modification of recommendations by Campbell et al. (5), Robertson et al. (12), and Blewett (4), with the goal of maximizing the

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proportion of fresh, viable oocysts (bovine genotype A) that would excyst (2). Optimizing excystation should minimize underestimation of the percentage of viable oocysts (2). Briefly, 100 μl of 2×10^7 to 4×10^7 oocysts/ml of suspension was mixed with 1 ml of HCl-acidified PBS (pH 3.8 at 37°C) and incubated at 37°C for 30 min. The oocyst suspension was then centrifuged at 11,600 × *g* for 10 min, the supernatant was removed, and the pellet was resuspended with 100 μl of Hanks balanced salt solution, 200 μl of 1% bovine bile (Ox Gall powder; Sigma) solution, and 50 μl of 0.44% $NaHCO_3$ solution. The solution was mixed with a wide-bore pipette and incubated overnight (approximately 17 h) at 37°C.

Enumeration of excystation percentages. A drop of thoroughly mixed postexcysted oocyst suspension was placed on a glass slide, coverslipped, and viewed under DIC optics (Nomarski) with an Olympus BX60 (Olympus America Inc., New York, N.Y.) microscope at 400× magnification. One hundred to 300 oocysts were examined per replicate of each protocol per trial, and the numbers of empty shells (ghosts), partially excysted oocysts, and intact (i.e., nonexcysted) oocysts were determined. The proportion of viable oocysts was calculated as $(E + P)/T$, where E is the number of empty oocysts, P is the number of partially excysted oocysts, and T is the total number of oocysts examined.

Isolation of nonexcysted oocysts. Nonexcysted oocysts were separated from partially excysted oocysts and ghosts with the protocol described by Arrowood and Sterling (1). Briefly, the postexcystation oocyst suspension was centrifuged at 1,500 × *g* for 15 min, the supernatant was removed, and the pellet was resuspended with Percoll solution (specific gravity, 1.04 g/ml) and centrifuged at 5,000 × *g* for 3 min. Aliquots of each layer were examined under DIC, and the bottom layer containing nonexcysted, intact oocysts was washed three times in deionized water by centrifugation at 1,500 × *g* for 10 min each. Nonexcysted oocysts were then enumerated with a phase contrast hemacytometer as above and serial dilutions were made from a stock solution containing 10^6 intact oocysts/ml. Intact oocysts were held for 24 h at 4°C prior to use as an inoculum.

Infectivity assay in neonatal CD-1 mice. The animal model used for determining infectivity of nonexcysted, intact oocysts was a modification of that of Neumann et al. (10). Female CD-1 mice with neonatal pups were purchased from Harlan (San Diego, Calif.), housed in cages fitted with air filters, and provided with feed and water ad libitum. Litters of 5-day-old neonatal mice were randomly assigned to one of the three excystation protocols and to one of the three doses of oocysts described below. Intragastric inoculations of oocysts were delivered in 100 μl of deionized water with a 24-gauge ball-point feeding needle. One hour prior to infection, the neonatal mice were removed from the dam to empty their stomachs for easier inoculation; following inoculation, the dam was returned to the pups. The dose given to each mouse was reconfirmed by reenumerating the oocyst suspension with a phase contrast hemacytometer, as described above.

Nonexcysted (intact) oocysts from trials 7, 8, and 9 (Table 1) were used to conduct three independent sets of in vivo experiments, A, B, and C, respectively (Table 2). To evaluate the infectivity of intact, nonexcysted oocysts derived from each of the three excystation protocols, a litter of CD-1 mouse pups was inoculated with either 50, 100, 500, or 5,000 intact oocysts/pup. In addition, for each dose in each experiment (A, B, or C) and for each protocol, each of a litter of pups that was used as a positive control group received an equivalent dose of freshly purified bovine oocysts (Table 2) and another litter of pups each received only 100 μl of distilled water (negative control). As another set of controls, 17 pups were each inoculated with 10^5 heat-inactivated oocysts (incubated at 70°C for 2 h) to determine if tissue homogenates from inoculated pups were made positive on epifluorescence microscopy due to detection of oocysts directly from the oral inoculum (false positive for infection status). Lastly, each of a litter of nine neonatal mice was inoculated with 10^5 sporozoites produced by excystation protocol III and held overnight at 4°C to determine if residual sporozoites inadvertently contained in our inoculum of postexcystation, intact oocysts could induce infection (false positive).

TABLE 1. Percent in vitro excystation resulting from three different protocols[b]

Trial	% Viable ($E + P/T$)[a]		
	Protocol I	Protocol II	Protocol III
1A	41.1 (88/214)	76.0 (146/192)	85.2 (98/115)
1B	60.7 (182/300)	80.7 (192/238)	91.5 (97/106)
2A	92.8 (285/307)	93.5 (144/154)	90.8 (129/142)
2B	91.5 (129/141)	92.8 (219/236)	92.0 (172/187)
3A	34.2 (41/120)	62.7 (74/118)	94.1 (95/101)
3B	43.8 (53/121)	59.0 (36/61)	96.3 (105/109)
4A	31.3 (70/224)	27.8 (60/216)	92.0 (185/201)
4B	29.6 (66/223)	24.5 (54/220)	93.0 (252/271)
5A	95.8 (184/192)	79.8 (174/218)	95.8 (138/144)
5B	94.7 (234/247)	81.9 (313/382)	94.0 (172/183)
6A	52.1 (100/192)	50.0 (99/198)	78.8 (160/203)
6B	53.1 (148/279)	48.0 (95/198)	78.1 (228/292)
7A	51.2 (215/420)	63.2 (187/296)	82.7 (235/284)
7B	ND[c]	ND[c]	ND[c]
8A	55.7 (108/194)	ND[c]	96.4 (189/196)
8B	38.7 (60/155)	ND[c]	97.5 (155/159)
9A	96.0 (215/224)	83.5 (198/237)	95.1 (252/265)
9B	99.0 (203/205)	80.8 (198/245)	97.3 (283/291)
Overall average	63.4 (2,381/3,758)	68.2 (2,189/3,209)	90.6 (2,945/3,249)

[a] Percent viable oocysts was calculated as the ratio of fully excysted or empty oocysts plus partially excysted oocysts ($E + P$) divided by the total number of oocysts examined (T).
[b] Protocol I is that of Black et al. (3), protocol II is that of Rennecker et al. (11), and protocol III is that of Atwill et al. (2).
[c] ND, not done.

Determination of infection. *C. parvum* infections in the mice were assessed by two methods: by staining homogenates of mouse intestinal tissue with a fluorescein isothiocyanate-labeled anti-*Cryptosporidium* immunoglobulin M antibody (Waterborne Inc., New Orleans, La.) and by histology performed by a board-certified veterinary pathologist (R. Moeller). Mice were euthanized by CO_2 asphyxiation 7 days after inoculation. A 5-mm portion of ileum, cecum, and colon was collected and immediately fixed in a 10% neutral-buffered

TABLE 2. Neonatal-mouse infectivity of intact *Cryptosporidium parvum* oocysts (bovine genotype A) isolated by one of three in vitro excystation protocols[a]

Trial[b]	Dose	% Infected (no. of infected pups/total no. of pups)									
		Protocol I		Protocol II		Protocol III		Positive control[c]		Negative control[d]	
		Homogenate	Histology	Homogenate	Histology	Homogenate	Histology	Homogenate	Histology	Homogenate	Histology
A	50	60 (6/10)	90 (9/10)	70 (7/10)	60 (6/10)	40 (4/10)	36.4 (4/11)	76.9 (10/13)	76.9 (10/13)		
A	500	100 (11/11)	100 (11/11)	100 (11/11)	100 (11/11)	83.3 (10/12)	66.7 (8/12)	100 (14/14)	100 (14/14)		
A	5000	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)	100 (12/12)	100 (12/12)	100 (12/12)	100 (12/12)		
A	0[f]									0 (0/11)	0 (0/11)
B	50	66.7 (8/12)	0 (0/12)	ND[e]	ND[e]	45.5 (5/11)	0 (1/11)	72.7 (8/11)	9.0 (1/11)		
B	500	ND[e]	ND[e]	ND[e]	ND[e]	92.3 (12/13)	23.1 (3/13)	100 (11/11)	72.7 (8/11)		
B	5000	100 (14/14)	100 (14/14)	ND[e]	ND[e]	100 (14/14)	100 (14/14)	100 (12/12)	100 (12/12)		
B	0[f]									0 (0/13)	0 (0/13)
C	50	70 (7/10)	70 (7/10)	75 (9/12)	66.7 (8/12)	58.3 (7/12)	75 (9/12)	77.8 (7/9)	77.8 (7/9)		
C	100	88.9 (8/9)	88.9 (8/9)	72.7 (8/11)	72.7 (8/11)	69.2 (9/13)	69.2 (9/13)	90.9 (10/11)	90.9 (10/11)		
C	500	100 (7/7)	100 (7/7)	100 (10/10)	50 (5/10)	100 (11/11)	100 (11/11)	100 (9/9)	100 (9/9)		
C	0[f]									0 (0/11)	0 (0/11)

[a] Protocol I was that of Black et al. (3), protocol II was that of Rennecker et al. (11), and protocol III was that of Atwill et al. (2). Two different methods were used to determine if neonatal mice were infected with *C. parvum* oocysts: staining of intestinal tract tissue homogenates and histopathology.
[b] Oocysts remaining intact after excystation trials 7, 8, and 9 (Table 1) were used to conduct three independent sets of neonatal-mouse experiments, A, B, and C, respectively.
[c] Fresh bovine genotype A oocysts which were not subject to excystation protocols.
[d] Neonatal mice inoculated with distilled water only.
[e] ND, not done.
[f] Inoculated with distilled water only.

formalin solution, processed using standard histopathology techniques, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin. Histologic sections of ileum, cecum, and proximal colon were examined for *C. parvum* oocysts attached to enterocytes under light microscopy at 100×, 200×, and 400× magnification. The remaining portions of the small and large intestine were suspended in 5 ml of deionized water and homogenized with a tissue homogenizer (IKA-Werke; GmbH & Co. KG, Staufen, Germany). The tissue homogenates were washed once in deionized water and centrifuged at 1,500 × *g* for 10 min, and the supernatant was removed. The pellets were resuspended in 10 ml of deionized water and filtered through a 20-μm-pore-size nylon net filter (Millipore Corp., Bedford, Mass.) fixed on a Swinnex (Millipore) holder. The filtrates were concentrated to 1 ml by centrifugation at 1,500 × *g* for 10 min and mixed on a vortex. Fifty microliters of the final homogenates was mixed with 50 μl of anti-*Cryptosporidium* monoclonal antibodies (Waterborne) and 2 μl of 0.5% Evans blue in PBS and incubated at room temperature for 45 min in a dark box. Three duplicate wet-mount slides were prepared from each sample, using 20 μl of reaction mixture per slide. The slides were examined by epifluorescence microscopy (Olympus America Inc.).

Data analysis. To determine which of the three protocols (2, 3, 11) were capable of maximizing the number of fresh oocysts that excyst, we used negative binomial regression, with the proportion of oocysts excysting modeled as an incidence rate ratio (8). The total number of oocysts determined to be viable (ghosts and partially excysted) functioned as the outcome variable, the type of protocol was the covariate, the total number of exposed oocysts functioned as an offset or exposure variable, and the trial id was set as a clustering variable to adjust *P* values for the potential lack of independence of oocyst excystation behavior within the trial (8).

We used logistic regression to compare the infectivity of intact, postexcysted oocysts from the three excystation protocols. We first set *p* equal to the proportion of oocysts classified as viable (ghost plus partially excysted). The logit [ln(*p*/1-*p*)] then functioned as the outcome variable, ln(dose) and method of excystation were set as covariates, and standard errors were estimated using a robust estimator to adjust *P* values for the potential lack of independence of oocyst excystation behavior within the trial (8). Infectivity results from histopathology and the tissue homogenates were analyzed separately and then compared against each other using McNemar's test to determine whether the dose-response curve for mouse infectivity was significantly different for the two infectivity assays.

Protocols I and II outlined by Black et al. (3) and Rennecker et al. (11) stimulated 63 and 68%, respectively, of fresh *C. parvum* oocysts to excyst (Table 1). In contrast, using the same batches of *C. parvum* oocysts and matched for age and storage conditions, our optimized protocol for in vitro excystation (2) stimulated a significantly higher amount of fresh bovine oocysts to excyst, 91% (95% confidence interval [CI], 87 to 95%) compared to 63 or 68% ($P < 0.01$). This result suggests that protocols I and II used in the original work to test the infectivity of intact oocysts may have inadvertently failed to excyst 25 to 30% of all possible excystable (i.e., viable) oocysts. Dosages of 50 and 500 intact oocysts were administered to neonatal mice in this earlier work, and up to 30% of these oocysts (15 and 150, respectively) were potentially excystable oocysts (presumably viable) rather than a pure population of nonexcystable (presumably nonviable) oocysts. These dosages (15 and 150 oocysts) of excystable oocysts could explain the levels of infec-

TABLE 3. Logistic regression model for the likelihood of neonatal-mouse infectivity of intact *Cryptosporidium parvum* oocysts (bovine genotype A) isolated from one of three in vitro excystation protocols

Factor	Infectivity measured with tissue homogenates			Infectivity measured with histopathology		
	Coefficient[a]	95% CI	Odds ratio	Coefficient[a]	95% CI	Odds ratio
Protocol[b]						
I	−0.48	−1.46, 0.50	0.62	−0.03	−0.79, 0.72	0.97
II	−0.45	−1.51, 0.61	0.63	−0.27	−1.11, 0.57	0.76
III	−1.45	−2.36, −0.55	0.23	−1.14	−1.85, −0.42	0.32
Positive control[c]	0		1.0	0		1.0
Ln (dose)	1.37	0.93, 1.81	3.94	0.80	0.60, 0.99	2.22
Constant	−4.14	−6.18, −2.10	0.02	−2.64	−3.71, −1.57	0.07

[a] Coefficient for the log odds or logit, defined as [ln(prevalence of infection/1−prevalence of infection)].
[b] Excystation protocol I was that of Black et al. (3), protocol II was that of Rennecker et al. (11), and protocol III was that of Atwill et al. (2).
[c] Referent category for the different excystation protocols, comprised of fresh bovine *C. parvum* oocysts.

tion originally observed in the neonatal mice by Neumann et al. (10). Using the infectious dose equations, we predicted that for the positive control oocysts (i.e., fresh and excystable oocysts) presented in Table 3 described below, a dose of 15 or 150 *C. parvum* oocysts of bovine genotype A would infect 4 of 10 or 9 of 10 neonatal CD-1 mice, respectively. These predicted levels of infection are very similar to the levels of infection observed by Neumann et al. (10), whereby one to two infections in 10 exposed mice were generated from a dose of 50 intact oocysts (containing 15 potentially excystable or viable oocysts) and six to nine infections were observed in 10 exposed mice from a dose of 500 intact oocysts (containing 150 potentially excystable or viable oocysts).

Using the oocysts that remained intact following excystation by either of these three protocols as the inoculum, we found that regardless of the choice of excystation protocol (I, II, or III) and regardless of the method used for determining infectivity (tissue homogenates or histopathology), intact oocysts were capable of initiating cryptosporidial infections in neonatal mice (Table 2). This observation is consistent with the finding by Neumann et al. (10) and therefore strongly suggests that excystation is an invalid measure of viability because oocysts failing to excyst, even when excystation is optimized, are infectious as determined by the neonatal CD-1 mouse model.

Data from the tissue homogenates indicated that the likelihood of infecting neonatal mice was significantly lower with intact oocysts isolated after excystation protocol III (2) than the likelihood of infecting neonatal mice with intact oocysts isolated after excystation protocols I or II (3, 11) (Table 3) (significant difference in the odds of mouse infection with oocysts remaining after protocol I versus protocol III, $P = 0.03$; protocol II versus protocol III, $P = 0.04$). In addition, the likelihood of infecting neonatal mice was significantly lower with intact oocysts isolated after excystation protocol III (2) than the likelihood of infecting neonatal mice inoculated with fresh oocysts (positive controls) ($P = 0.002$). In contrast, the likelihood of infection of neonatal mice was not significantly different for intact oocysts isolated after excystation protocols I or II compared to that for fresh oocysts (positive controls) (Table 2). This lack of difference between the infectious potential of fresh oocysts and intact oocysts isolated after protocols I and II is consistent with the findings of Neumann et al. (10), who also observed a lack of difference in the proportion of mice infected with original fresh stock and those infected with nonexcysted, intact oocysts. The pattern of lower infectivity for intact oocysts resulting from the optimized excystation protocol III compared to that for either fresh oocysts or oocysts remaining after protocol I or II was the same for the data generated by histopathology (Table 3). We can speculate that either the intact, nonexcysted oocysts isolated after protocol III are in fact less infectious for neonatal mice due to dysfunctionalities with the process of excystation or some other physical or biochemical problem associated with asexual or sexual multiplication or they are the result of the longer incubation time associated with protocol III (17 h at 37°C) than with protocols I and II (3 h at 37°C). No infection was observed in the neonatal mice among our negative controls that were inoculated with either distilled water, 10^5 heat-inactivated oocysts, or 10^5 sporozoites that had been held overnight at 4°C.

Our results indicate that the method of using tissue homogenates coupled with epifluorescence microscopy for determining the infection status of inoculated neonatal mice was significantly more sensitive than histopathology. When a McNemar's test was used to determine the equality of the infection rates for paired dependent data (i.e., two methods were applied to the same set of mouse tissues), the odds for detecting an active infection in a neonatal mouse via histopathology was 0.42 compared to the odds for detecting an active infection in a neonatal mouse via tissue homogenates (odds ratio, 0.42; 95% CI, 0.26 to 0.66; $P = 0.001$). Therefore, using tissue homogenates as a more valid standard to measure infection status among inoculated CD-1 neonatal mice, the extrapolated 50% infective doses for fresh bovine genotype A oocysts (positive controls) and intact oocysts isolated after excystation protocols I, II, and III were 20.4, 32.9, 32.1, and 87.3, respectively. Based on these results, we would agree with Neumann et al. (10) that intact *C. parvum* oocysts isolated after excystation are infectious for neonatal mice. This finding indicates that excystation should not be considered a valid measure of *C. parvum* viability, given that it is biologically implausible for oocysts to be nonviable and yet infectious.

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