Virulence of Three Distinct *Cryptosporidium parvum* Isolates for Healthy Adults

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The infectivity of three *Cryptosporidium parvum* isolates (Iowa [calf], UCP [calf], and TAMU [horse]) of the C genotype was investigated in healthy adults. After exposure, volunteers recorded the number and form of stools passed and symptoms experienced. Oocyst excretion was assessed by immunofluorescence. The ID_{50} differed among isolates: Iowa, 87 (SE, 19; 95% confidence interval [CI], 48.67–126); UCP, 1042 (SE, 1000; 95% CI, 0–3004); and TAMU, 9 oocysts (SE, 2.34; 95% CI, 4.46–13.65); TAMU versus Iowa, $P = .002$ or UCP, $P = .019$. Isolates also differed significantly ($P = .045$) in attack rate between TAMU (86%) and Iowa (52%) or UCP (59%). A trend toward a longer duration of diarrhea was seen for the TAMU (94.5 h) versus UCP (81.6 h) and Iowa (64.2 h) isolates. *C. parvum* isolates of the C genotype differ in their infectivity for humans.

Cryptosporidium parvum, a coccidian protozoan of worldwide distribution, is frequently found in surface waters and is now a leading cause of waterborne outbreaks in the United States [1, 2]. Exposure in healthy individuals results in transient infection that may be asymptomatic or result in self-limited diarrhea. In individuals coinfected with the human immunodeficiency virus (HIV), the spectrum of disease may range from asymptomatic infection or self-limited disease early in the course of HIV to a severe life-threatening illness in those with advanced AIDS [3].

A number of host, environmental, and parasite-specific variables are cofactors that interplay in the prevalence of *C. parvum* in humans. It is well known that among other factors, host immunity and intensity of water contamination have an impact in disease manifestation. There is also evidence that parasite-related factors might have an impact on intensity of infection and/or severity of clinical manifestations. For instance, at comparable levels of immunosuppression, individuals with AIDS can show a high variability in oocyst excretion, clinical manifestations, and response to therapy [4–6]. In addition, isolates from patients with AIDS show differences in pathogenicity when passaged in calves [7]. Other evidence for isolate-specific differences are provided by studies that have documented differences in mice infectivity for isolates derived from humans [8], in protein composition [9], and in immunoreactivity [10].

It has long been speculated that clinical severity and/or attack rates for *C. parvum* infections might be related to genotype and/or specific virulence differences among isolates. In recent years, progress has been made in determining the population structure of *C. parvum.* Analysis of genotypic heterogeneity in single and multiple loci has identified 2 major genotypes, designated H and C (also known as 1 and 2, respectively), that result in 2 independent human transmission cycles [11–16]. Isolates that belong to the genotype H, or 1, are associated with human and nonhuman primate infection, whereas the genotype C, or 2, is associated with infection in calves and humans. Using single-locus polymorphism, Patel et al. have recently described *C. parvum* genotypes in sporadic infections, waterborne outbreaks, and livestock samples. In that study, 59% of sporadic human infections were caused by genotype H and 35% by genotype C. In contrast, 96% of human clinical samples linked to 2 waterborne outbreaks were caused by the H type. All infections in livestock where associated with genotype C. Only a small minority (<5%) of patients showed coinfection with both genotypes [17].

The objectives of this study were to investigate differences in infectivity of 3 distinct *C. parvum* isolates belonging to the C genotype. The ID_{50} and clinical outcome (i.e., attack rate, ratio of infected to uninfected, duration of diarrhea, and relapse

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rate) were determined for each isolate after experimental infection of healthy adults.

Materials and Methods

Isolates studied. Three geographically distinct isolates were used in this study. The Iowa isolate has been used by our group in earlier infectivity studies in subjects with [18] and without [19, 20] prior exposure to *C. parvum* as determined by anti-*C. parvum* ELISA, as well as in subjects who received a second challenge [21]. This isolate was derived originally from a calf and was collected by Dr. Harley Moon (University of Iowa, Ames). It has been repeatedly passaged in calves in the laboratory of Dr. Charles Sterling (University of Arizona, Tucson). The second isolate, designated UCP, was also derived from a calf and was originally obtained by Dr. Elizabeth Ungar (Uniformed Services University of the Health Sciences, Bethesda, MD). This isolate has been passaged in calves at least 10 times in the laboratory of Dr. Joseph H. Crabb (ImmuCell Corp, Portland, MA) and has been used in a clinical study that investigated the prophylactic effect of a hyperimmune bovine colostrum preparation [22]. The third isolate, designated TAMU, was collected from a veterinary student who was exposed during necropsy of an infected foal. The sample was obtained by Dr. Karen Snowden (Texas A&M University, College Station). The TAMU isolate was then passaged in calves by Dr. Charles Sterling at the University of Arizona in the same fashion as the Iowa isolate. Oocysts were propagated in newborn calves and purified according to the method of Arrowood and Sterling [23]. In the case of the UCP isolate, a 1-min exposure of the semipurified preparations to 0.5% (v/v) sodium hypochlorite was followed by quenching with sodium thiosulfate. Prior to use in volunteers, the oocyst preparations were cultured for bacteria, mycobacteria, and fungi. Transmission electron microscopy and a variety of cell lines (WI-38, MRC-5, RKC, A549, PRMK 1 and 2) were used for adventitious virus detection [19]. In parallel studies conducted in mice, none of the 3 isolates showed decreases in infectivity over time, and in the case of UCP oocysts, hypochlorite-treated oocysts showed mouse infectivity similar to untreated oocysts (data not shown).

Prior to use, oocyst preparations were washed twice in 10 mL of cold, sterile PBS, pH 7.2, to remove potassium dichromate, resuspended in cold, sterile PBS, and serially diluted. Oocysts were counted by using a hemocytometer and were adjusted to the desired dose. A minimum of 5 additional counts was done to yield an accurate estimate of the inoculum. Volunteers ingested oocysts in gelatin capsules, as described elsewhere [19]. Oocysts were ingested within 14–51 days of calf passage and in all cases were shown to have an excystation rate of 85% or greater at the time of challenge. Actual oocyst concentrations delivered to the volunteers were typically within a median coefficient of variance of ≤5% of the target dose. Volunteers received oocysts at doses of 30–10^6 oocysts in the case of the Iowa isolate, 500–10^4 for the UCP isolate, and 10–500 for the TAMU isolate. Volunteers were allocated to the different dose levels studied by use of a mathematical model described elsewhere [24]. The final distribution is shown in table 1.

Human subjects, evaluation of stools, and definition of terms. Volunteers who agreed to participate were asked to provide informed consent and a detailed medical history. Prior to oocyst challenge, participants underwent a physical examination and extensive laboratory studies for general health assessment, as described elsewhere [19]. Volunteers collected all stools passed for the first 2 weeks of the study and two 24-h collections thereafter, for a total of 6 weeks after challenge. A diary was kept by each volunteer describing the time and characteristic of all stools passed and symptoms experienced. All stools collected were examined for the presence of *Cryptosporidium* by direct immunofluorescence assay [19]. Stools from all episodes of diarrhea were cultured for enteric bacterial pathogens, including *Shigella, Salmonella, Campylobacter, Aeromonas,* and *Plesiomonas.*

Table 1. Number of volunteers for each dose of 3 geographically distinct isolates of *Cryptosporidium parvum* oocysts.

	No. of volunteers		
Dose, no. oocysts	Iowa (n = 29)	UCP (n = 17)	TAMU (n = 14)
10	0	0	3
30	5	0	3
100	8	0	3
300	3	0	0
500	6	5	5
1000	2	3	0
5000	3	5	0
10,000	1	4	0
>10,000	1	0	0

Diarrheal illness was defined as any one of the following: the passage of 3 unformed stools in 8 h, the passage of 4 or more unformed stools in 24 h, or the passage of >200 g/24 h of unformed stools accompanied by at least 1 enteric symptom. Enteric symptoms were defined as the occurrence of nausea, vomiting, abdominal pain, flatulence, tenesmus, fecal urgency, or incontinence in the setting of passing unformed stools not meeting the above criteria. A confirmed infection was defined as the presence of fecal oocysts in the stool 36 h or more after challenge. Presumed infection was defined as having a diarrheal illness or enteric symptoms without demonstrated oocysts within 30 days after challenge. The term *presumed* is used because of the limitation of direct immunofluorescence assay (DIFA) in detecting <10,000 oocysts/mL [25]. The time to onset of diarrhea was determined as the number of days that elapsed between oocyst challenge and passage of the first unformed stool after which illness was declared. The duration of diarrhea in hours was defined as described elsewhere [26]. The attack rate was defined as the percentage of individuals who developed diarrhea after oocyst exposure. The ratio of infected to uninfected volunteers was defined as the number of individuals who developed diarrhea and/or oocyst excretion after exposure divided by the total number of subjects challenged with each isolate.

Statistical methods. The dose response curves for each of the isolates were graphed by use of the method described by Reed and Muench [27]. For statistical comparisons, the data were analyzed by linear regression fit on the basis of least-squares estimation. Using this method, we determined ID_{50}, standard error, and 95% confidence intervals (CIs). Assuming normality, we determined comparisons between isolates using z scores and t tests. A Kruskal-Wallis nonparametric analysis of variance test was used to evaluate

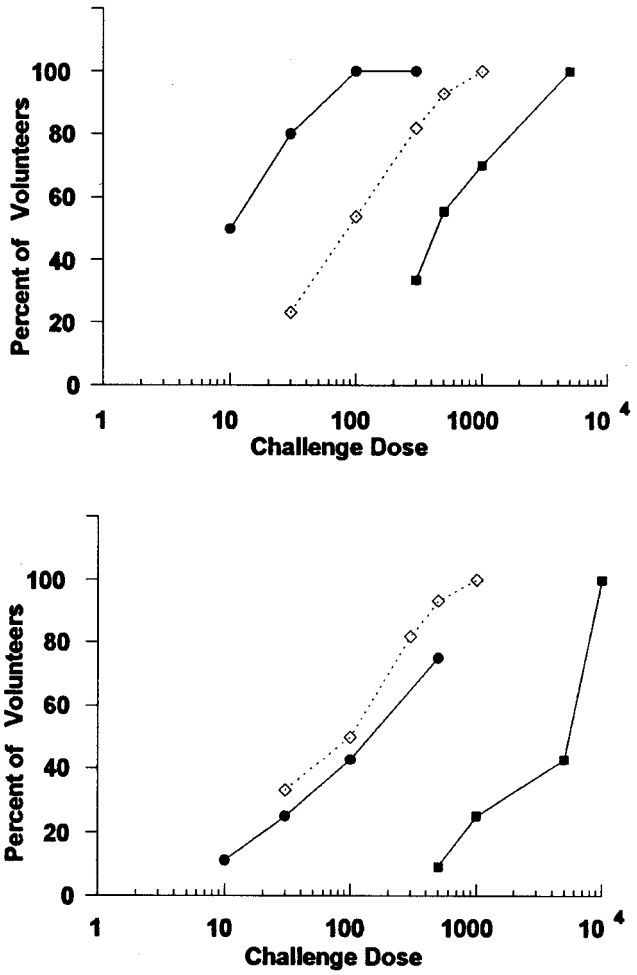

Figure 1. Infectious dose curves for 3 *Cryptosporidium parvum* isolates belonging to the C genotype. ♦, Iowa isolate; ■, UCP isolate; ●, TAMU isolate. *A,* cumulative percentage of volunteers developing diarrhea and or excreting oocysts is plotted according to the challenge dose received. *B,* cumulative percentage of individuals only excreting oocysts. Viability and age of production of oocysts were similar.

the relationship between challenge dose and clinical outcomes (onset, duration, severity, or number of diarrheal episodes) or infection parameters (onset of oocyst excretion and total number of oocysts passed). The differences in the proportion of presumed infections from each isolate were evaluated by χ^2 contingency tables. Differences in the time to onset of illness and duration of illness were compared by the Kaplan and Meier methods. Data were analyzed with Statview version 4.1 for MacIntosh.

Results

The general demographic characteristics of the participating volunteers were similar in regard to sex, age, and anti-*C. parvum* status (all negative) for volunteers participating in each of the dose-response experiments. A total of 29 volunteers was challenged with the Iowa isolate, 17 with the UCP isolate, and 14 with the TAMU isolate. In parallel studies conducted in mice, none of the 3 isolates showed decreases in infectivity over time, and in the case of UCP oocysts, hypochlorite-treated and untreated oocysts showed similar infectivity. Furthermore, the rates in excystation decline and mice infectivity in treated and untreated oocysts did not differ after storage (data not shown).

Infectious dose curves were plotted by use of data generated from subjects classified as having a presumed infection (figure 1*A*) and those only with documented oocyst shedding (figure 1*B*). Using presumed infection as the outcome variable, we calculated the ID_{50} value for the IOWA isolate to be 87 oocysts (SE, 19; 95% CI, 48.67–126), 1042 oocysts for the UCP isolate (SE, 1000; 95% CI, 0–3004), and 9 oocysts for the TAMU isolate (SE, 2.34; 95% CI, 4.46–13.65). Pairwise comparisons showed significant differences between the TAMU and Iowa ($P = .002$) or UCP isolates ($P = .019$). No difference was found between the UCP and Iowa isolates. Note that at a dose of 300 oocysts, all of the TAMU, but only 80% of the Iowa and 55% of the UCP participants, were presumed to be infected. Alternatively, when detectable oocyst excretion was used as the defining parameter (figure 1*B*), the ID_{50} value was 74.5 oocysts (SE, 20.56; 95% CI, 34–114) for the Iowa isolate, 2788 oocysts (SE, 4298.74; 95% CI, 0–11214) for the UCP isolate, and 125 oocysts for the TAMU isolate (SE, 27; 95% CI, 72.62–179). However, the greater variation in the data did not allow for statistically significant differences. The duration of diarrhea and percentage of volunteers with a symptomatic relapse was similar in the 3 groups of volunteers studied, as shown in table 2. No

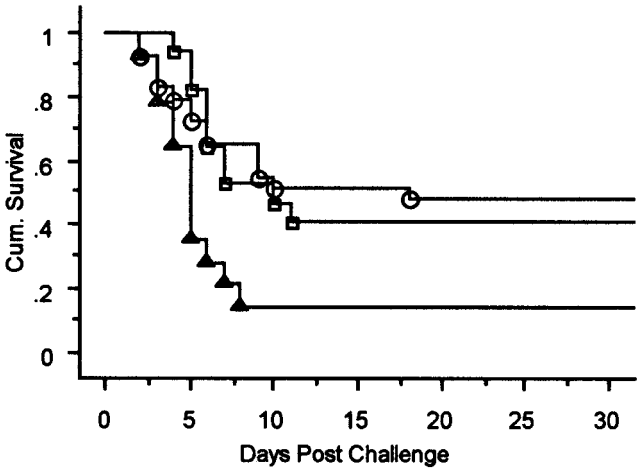

Figure 2. Time to onset of diarrhea as determined by Kaplan-Meier survival curves in healthy volunteers after exposure to *Cryptosporidium parvum* isolates of the C genotype. Plot represents the proportion of subjects that did not experience diarrhea. □, Iowa isolate; ○, UCP isolate ; △, TAMU isolate. $P = .02$ for TAMU vs. UCP and/or Iowa.

Table 2. Clinical outcomes for healthy volunteers after exposure to 3 geographically distinct isolates of *Cryptosporidium parvum.*

Parameter	Isolate			
	Iowa (n = 29)	UCP (n = 17)	TAMU (n = 14)	P[a]
Attack rate, %	52	59	86	<.055
Duration of diarrhea, h ± SD (range)	64.2 ± 67.43 (6–223)	81.6 ± 59.68 (6–193)	94.5 ± 66.7 (6–195)	NS
Relapse rate, %	18	46	58	NS
Total no. unformed stools, mean	6.7	8.8	8.2	NS
Total weight of stools, mean grams	936	818	1278	NS

NOTE. NS, not significant.
[a] TAMU vs. Iowa or UCP.

differences were observed in total number of unformed stools or diarrheal stool weight. In contrast, a higher attack rate (P = .04) was seen in the group of volunteers exposed to the TAMU isolate than in volunteers exposed to UCP or Iowa oocysts.

During the study, 12 (86%) of 14 volunteers who received the TAMU isolate developed diarrhea, as did 15 (52%) of 29 who received the Iowa isolate and 10 (59%) of 17 who received the UCP isolate. Similarly, an earlier onset of disease was seen in subjects exposed to the TAMU isolate: the mean onset of illness was 5 days, whereas for those who recevied the Iowa isolate, it was 9 days, and for those who received the UCP isolate, 11 days (log rank, $P < 0.01$; figure 2). The proportion of subjects excreting oocysts was similar regardless of the isolate to which they were exposed. Eighteen (62%) of 29 subjects who received the Iowa isolate excreted oocysts, 7 (41%) of 17 who received the UCP isolate, and 7 (50%) of 14 who received the TAMU isolate. Similarly, the proportions of individuals with diarrheal illness in whom detectable oocysts were not identified were also similar (i.e., 50% of subjects in the TAMU group, 40% in the UCP group, and 44% in the Iowa group). The onset, duration, and intensity of oocyst excretion were similar for all groups (table 3).

Discussion

Three epidemiologic components have traditionally been linked to the incidence and severity of cryptosporidiosis (summarized by Meinhardt et al. [28]). They include environmental factors, host immunity, and parasite dynamic factors. Some of the environmental factors include the degree of water contamination with *Cryptosporidium,* the homogeneity of parasite dispersion, and environmental stressors, such as salinity and temperature [29]. The susceptibility of the host also contributes to clinical manifestations. For instance, previous studies in healthy adults have shown that individuals without prior exposure, as determined by absence of anti–*C. parvum*–specific antibodies in serum by ELISA, are more susceptible to low doses of oocysts than those with ELISA seroreactivity [18]. Similarly, individuals who are reactive to 17 and 27 kDa antigens on immunoblot are less likely to exhibit clinical disease [30, 31]. Studies have also shown that when anti–*C. parvum*–negative volunteers are reexposed, they may still develop illness but shed fewer oocysts, suggesting the development of only partial immunity [21]. This is in contrast to the experience of subjects living in endemic locations, where constant exposure to oocysts takes place and asymptomatic excretion is common [32].

Parasite dynamic factors that may contribute to infectivity include selectivity for human hosts, differences in susceptibility to environmental stressors, drug susceptibility, and variability in virulence. Examples of host selectivity are the preference of non-*parvum* cryptosporidia for certain animal species. Genetic analysis of isolates derived from humans and calves has resulted in the clustering of *C. parvum* isolates into 2 major groups: genotype C isolates infect humans and calves, whereas a second genotype (genotype H) selectively infects humans [11–16] and nonhuman primates. The latter is thought to be the genotype isolated most frequently from individuals with AIDS-associated cryptosporidiosis [33] and in sporadic infection [17] with little evidence for recombination. Analogous genotype clustering has been observed in *Toxoplasma* [34, 35].

In this study, the infectivity of 3 well-characterized genotype C isolates was studied. The study was conducted in healthy volunteers in a controlled fashion. Thus, the relative contributions of environmental variables and host immunity were kept constant. That is, volunteers received controlled numbers of oocysts prepared in the same fashion and with similar excystation rates; all subjects were healthy, serologically negative for *C. parvum,* and all had normal immunologic parameters. The results presented here suggest that geographically diverse isolates of the C genotype differ in their ability to produce disease. This has been shown by differences in infectious dose, as well as in other parameters, such as the duration of the prepatent period and proportions of individuals showing symptoms. Of interest, the TAMU isolate differed from the other two isolates in two important aspects. First, this isolate was obtained from a healthy subject who was, in turn, infected from a foal. The isolate was then passaged in calves and used for volunteer studies; thus, it has been passaged in calves significantly fewer times than the other isolates. It is plausible that its relatively more recent passage in a human or horse may select for or induce virulence factors that result in a more clin-

Table 3. Infection parameters for volunteers challenged with 3 distinct *Cryptosporidium parvum* isolates.

Parameter	Isolate			
	Iowa (n = 18)	UCP (n = 7)	TAMU (n = 7)	P[a]
Onset of shedding, mean days after challenge	7.7	7	4	NS
Duration of shedding, mean days	8.4	3.3	3.4	NS
Intensity of oocyst excretion, log mean ± SD[a]	6.32 ± 2.28	6.10 ± 2.26	6.68 ± 2.16	NS
Ratio of infections confirmed vs. presumed	0.95[b]	0.46	0.46	<.01[a]

NOTE. NS, not significant.
[a] Total oocysts excreted for the duration of the study.
[b] Iowa vs. TAMU or UCP.

ically severe course of illness. Alternatively, repeated passage of the other 2 isolates may have resulted in some attenuation. Nevertheless, our data support the in vivo and in vitro observations that isolates show variability in their infectivity in animal models, in the induction of transmembrane potential, and in the release of lactic dehydrogenase in Caco-2 cell monolayers [33].

Several putative virulence factors have been identified in *Cryptosporidium.* A membrane aminopeptidase involved in excystation has been described elsewhere [36], as have a cysteine proteinase [37] and a hemolysin [38]. Factors that are thought to be of importance in infectivity include molecules that mediate adherence and cell attachment, such as lectins [39, 40], Gp 900, a rhoptry glycoprotein of high molecular weight [41], TRAP-C, a protein with similarity to thrombospondin related adhesion molecules [42], and others, such as 15 and 25 kDa proteins associated with parasite gliding [43]. The presence of an enterotoxin has also been suggested by in vitro studies [44, 45], but a definite molecule has yet to be identified. The characterization of these factors and others and the mechanisms that regulate them should help in determining their role in virulence and assist in the development of isolates that are antigenic but attenuated, resulting in potential vaccine candidates. A more detailed genotypic, immunologic, and phenotypic analysis is under way for these three isolates.

The ID_{50} for the Iowa and Texas isolates were found to be similar when the detection of oocysts by DIFA was used as the defining criteria for infection. This may reflect the fact that individuals exposed to the Iowa isolate excreted oocysts for a longer period of time and therefore were more likely to have oocysts identified. As in previous studies, oocysts could not always be identified by direct immunofluorescence in a significant proportion of subjects with diarrhea. This was most likely caused by lack of sensitivity of DIFA rather than a lack of infection because oocysts could be identified in several individuals by flow cyotmetry (data not shown). In this study, the overall sensitivity of DIFA was only 80%. Of interest, oocysts were detected in 85% of those with diarrhea caused by the Iowa isolate but only in 60% of those receiving the UCP isolate and 54% in the case of the TAMU isolate. These observations also support the notion that the infectious dose for the TAMU isolate is lower than that for the other isolates. Also of note, the subjects in the TAMU group received considerably fewer oocysts and yet developed a higher attack rate and had a higher number of oocysts produced. Potential explanations for this observation are differences in virulence intrinsic to the isolate or a more effective amplification at lower inocula. This latter phenomenon was observed previously with the Iowa isolate at the lower inocula [20]. Studies with magnetic beads coated with *Cryptosporidium* specific antibodies to capture oocysts, flow cytometry, and the polymerase chain reaction are being conducted to improve oocyst detection.

Another limitation in this study is the sensitivity of ELISA in detecting prior exposure. With this serologic method, it is difficult to exclude with complete certainty that individuals had never been exposed to *C. parvum.* They may have had prior exposure, but lost the serum antibody over time or may have had an exposure that was not sufficient for antibody generation and thus may represent a population of subjects with heterogeneous past exposures and variable susceptibilities to infection. However, our previous observation that seropositive individuals are much less susceptible to infection would support the fact that ELISA serostatus can be used as a marker for relative susceptibility to future oocyst exposures. Furthermore, the dose-dependent effect seen for each one of the isolates suggests that parasite factors contribute more than host factors in this population to differences in illness. One other limitation is the fact that the 3 isolates studied here belong to the C genotype, a genotype that has zoonotic potential. It is unknown whether oocysts belonging to the H genotype (that preferentially infect primates) would show similar infectivity and/or clinical manifestations. Finally, it is unlikely that the hypochlorite exposure used to treat the UCP oocysts had a significant impact. Indeed, significant differences were seen between TAMU and Iowa isolates that were prepared in the exact same manner. Further, previous studies have shown that a short exposure to hypochlorite does not significantly decrease infectivity in animals [46].

In summary, the data presented here confirm our prior observations that *C. parvum* oocysts derived from calves can produce infection in healthy humans at a low inoculum. Our data also provide clinical observations suggesting that *C. parvum*

isolates differ in their infectivity for humans. These data are consistent with prior observations of pathogenic variability seen in vitro.

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