

Identification of 5 Types of *Cryptosporidium* Parasites in Children in Lima, Peru

Lihua Xiao,¹ Caryn Bern,¹ Josef Limor,¹
 Irshad Sulaiman,¹ Jacquelin Roberts,¹
 William Checkley,^{2,4} Lilia Cabrera,⁴ Robert H. Gilman,³⁻⁵
 and Altaf A. Lal¹

¹Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; ²Northwestern University Medical School, Chicago, Illinois; ³Johns Hopkins University School of Public Health, Baltimore, Maryland; ⁴Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura (PRISMA), and ⁵Universidad Peruana Cayetano Heredia, Lima, Peru

Cryptosporidium parvum is usually considered to be the pathogen responsible for human cryptosporidiosis. We genotyped *Cryptosporidium* in 132 stool specimens from 80 Peruvian children, representing 85 infection episodes, using techniques that differentiate *Cryptosporidium* species and *C. parvum* genotypes. Five types of *Cryptosporidium* were identified: *C. parvum* human (67), bovine (8), and dog (2) genotypes, *C. meleagridis* (7), and *C. felis* (1). Twenty-five (29%) of the 85 infection episodes were associated with diarrhea. There was no significant difference in age, antecedent stunting, percentage with diarrhea, or duration of diarrhea for episodes with human genotype, compared with those of zoonotic *Cryptosporidium*. Duration of oocyst shedding was longer for human genotype than for zoonotic *Cryptosporidium* (mean, 13.9 days and 6.4 days, respectively; $P = .004$). Serum samples from 8 children with *C. meleagridis*, *C. felis*, or *C. parvum* dog genotype were tested for anti-human immunodeficiency virus (HIV) type 1 antibodies; all were found to be negative. Contrary to common belief, novel *Cryptosporidium* species and *C. parvum* genotypes can infect HIV-negative children.

Cryptosporidiosis is a common cause of diarrheal disease in humans and other animals [1]. *Cryptosporidium parvum* has commonly been considered the only etiologic agent of cryptosporidiosis in immunocompetent persons. This conclusion has been based on the morphologic similarity and cross-transmission potential of *Cryptosporidium* oocysts found in human infections and known *C. parvum* oocysts from calves, as well as a belief in the monospecific nature of *Cryptosporidium* parasites [2–4]. Results of most recent genotyping studies support this view. To date, only 2 genotypes of *C. parvum* have been identified in immuno-

competent humans: the human genotype (genotype 1 or anthroponotic genotype), exclusively found in humans, and the bovine genotype (genotype 2 or zoonotic genotype), which infects humans, ruminants, and some other animals [5, 6]. However, the genotyping tools used thus far in most studies have only been capable of detection and differentiation of the human and bovine genotypes.

Recent studies indicate the existence of multiple *Cryptosporidium* species, including several that are morphologically similar and genetically closely related to *C. parvum* [7]. Using newer molecular tools with a broader capability to detect and differentiate strains, 2 novel *Cryptosporidium* parasites, *C. felis* and *C. meleagridis*, as well as *C. parvum* dog genotype, were detected in patients with AIDS [8, 9]. Furthermore, oocysts with morphology suggestive of *C. muris* were detected in specimens from 2 Indonesian children who did not have gastroenteritis [10]. These and other recent findings call into question the accepted view that human cryptosporidiosis is caused by a single species of parasite and have motivated a reexamination, using new molecular techniques, of the public health importance of *Cryptosporidium* parasites other than *C. parvum*.

In this study, we genotyped *Cryptosporidium* parasites from children in Lima, Peru, who participated in a 3-year cohort study of diarrheal disease and enteric pathogens, using a molecular tool that differentiates among a range of *Cryptosporidium* species and *C. parvum* genotypes. We then sought epidemiologic and clinical correlates of the various *Cryptosporidium* strains found.

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Written informed consent was obtained from the parent or guardian of all participating children. The original cohort study was approved by the institutional review boards of the Johns Hopkins University School of Hygiene and Public Health and Asociación Benéfica PRISMA. The *Cryptosporidium* genotyping study, including human immunodeficiency virus testing, was approved by the Centers for Disease Control and Prevention Institutional Review Board.

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Reprints or correspondence: Dr. Lihua Xiao, Division of Parasitic Diseases, Mailstop F-12, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, N.E., Atlanta, GA 30341-3717 (lax0@cdc.gov).

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Subjects, Materials, and Methods

Study subjects. The field work for this study was conducted in the peri-urban *pueblo joven* (shantytown) of Pampas de San Juan de Miraflores, 25 km from the center of Lima. This community of ~40,000 inhabitants was settled predominantly in the 1980s by immigrants from rural areas. In recent years, immigration to the community has slowed, and general living conditions have improved. When the cohort study began, 97% of houses had electricity, 48% had toilets, and 64% had a household water connection (R.H.G., unpublished data). Although human immunodeficiency virus (HIV) and AIDS have been recognized as a public health problem in Peru, with ~1000 cases per year reported in surveillance data (National AIDS and STD Control Program, Ministry of Health, Lima, Peru), the prevalence in the community of Pampas de San Juan de Miraflores, only 5 (3.7%) HIV infections were detected, all in adults (G. Soto, personal communication).

We conducted this study using stool specimens and data from a cohort of children who participated in a longitudinal study of diarrheal disease. The children were monitored from April 1995 to December 1998. At the time of recruitment, field workers administered a questionnaire that collected data regarding household characteristics, including the type of housing, sanitary facilities, water source, and the presence of animals. Children's height and weight were measured monthly. Field workers visited the household daily throughout the follow-up period to compile a daily record of the primary caretaker's opinion on whether diarrhea was present, the number of bowel movements, and the consistency of stools (liquid, semiliquid, or formed). Stool specimens were collected weekly from all children on the first day of a diarrheal episode and, when one of the pathogens of interest was detected, daily until specimens were negative. Stool specimens were transported without preservative. An aliquot from each specimen was processed by using the standard formalin-ethyl acetate concentration procedure and examined microscopically for *Cryptosporidium* oocysts on modified acid-fast Ziehl-Neelsen stained slides [11] in the pathology laboratory of the Universidad Peruana Cayetano Heredia (Lima). The intensity of infection was quantified by the number of oocysts counted in a 20- μ l volume of concentrated stool specimen: 1+ (1–50 oocysts), 2+ (51–150 oocysts), and 3+ (>150 oocysts). One or more aliquots of each specimen were frozen for future studies. The current study focused only on the subset of children whose stool specimens contained *Cryptosporidium* at least once and from whom ≥ 1 *Cryptosporidium*-positive specimens was available for molecular study.

Stool samples and DNA extraction. Stool samples were stored in Lima, Peru, for 1–4 years at -20°C before DNA extraction. DNA was extracted directly from frozen fecal specimens (<0.5 g/sample) by phenol-chloroform extraction, as described elsewhere [12, 13]. To remove polymerase chain reaction (PCR) inhibitors, DNA extracted from some stool samples was further purified by passing the suspension through QIAamp DNA Mini isolate columns (Qiagen).

Genotype analysis. All DNA samples were examined initially by a PCR–restriction fragment length polymorphism (RFLP) technique, which detects and differentiates all currently recognized

Cryptosporidium species and *C. parvum* genotypes [12, 13]. In this method, an 834-bp segment of the *Cryptosporidium* small subunit (SSU) rRNA gene was amplified by nested PCR. Primers and amplification conditions used in this study are described elsewhere [13], except that the reverse primer used in the primary PCR was 5'-CCCATTTCCTTCGAAACAGGA-3'. Genotype identification was made by restriction digestion of the secondary PCR product with *SspI* and *VspI*. Each sample was analyzed at least twice in 2 independent PCR-RFLP analyses. The identification of genotypes other than the *C. parvum* human genotype was further confirmed by sequence analysis of the PCR products on an ABI 377 auto-sequencer (Applied Biosystems). In addition, the samples that were positive for unusual genotypes were also analyzed by PCR and by sequencing of the 70-kDa heat-shock protein (hsp70) gene [14].

HIV-1 testing. For a subset of children with unusual *Cryptosporidium* genotypes, serum specimens were obtained during the period of cohort follow-up. The serum specimens were tested for the presence of antibodies against HIV-1 and HIV-2 at the Centers for Disease Control and Prevention (Atlanta) by using an HIV peptide EIA kit (BioRad Laboratories).

Epidemiologic analysis. We defined a day with diarrhea as a 24-h period during which the child was reported to have ≥ 3 liquid or semiliquid stools and, in addition, was thought by his or her primary caretaker to have diarrhea. An episode of diarrhea was considered to end when the child had ≥ 3 days that did not meet the criteria for a day with diarrhea. An episode of cryptosporidiosis was defined by ≥ 1 stool specimen that was positive for *Cryptosporidium*. An episode of *Cryptosporidium* infection was considered to end on the last day of oocyst detection, followed by ≥ 8 weeks without oocyst detection. For the analysis of epidemiologic correlates of *Cryptosporidium* genotypes, we compared infections with *C. parvum* human (anthroponotic) genotype with infections with all other genotypes, which we refer to in this paper as “zoonotic genotypes.” This designation is not intended to indicate that transmission of the particular infection episodes reported here was necessarily from animal to human but recognizes that *C. parvum* bovine and dog genotypes, *C. felis*, and *C. meleagridis* have all been reported as the cause of infection in animals and have zoonotic transmission potential.

Data were analyzed in SAS for Windows, version 6.12 (SAS Institute). Significance testing was performed by using a pooled or ranked Student's *t* test for continuous variables and by using χ^2 or Fishers exact test for categorical variables. When appropriate, SUDAAN version 7.5 (Research Triangle Institute) was employed to account for correlation among multiple measurements from the same person.

Results

***Cryptosporidium* genotypes.** A total of 271 microscopy-positive stool specimens from 119 children were available for molecular analysis. Successful PCR amplification of the *Cryptosporidium* SSU rRNA gene was accomplished for 132 stool specimens from 80 children, representing 85 episodes of *Cryptosporidium* infection. The accidental freeze-thawing of samples several times during storage was contributory to the low number of positive results of PCR amplification. RFLP analysis of

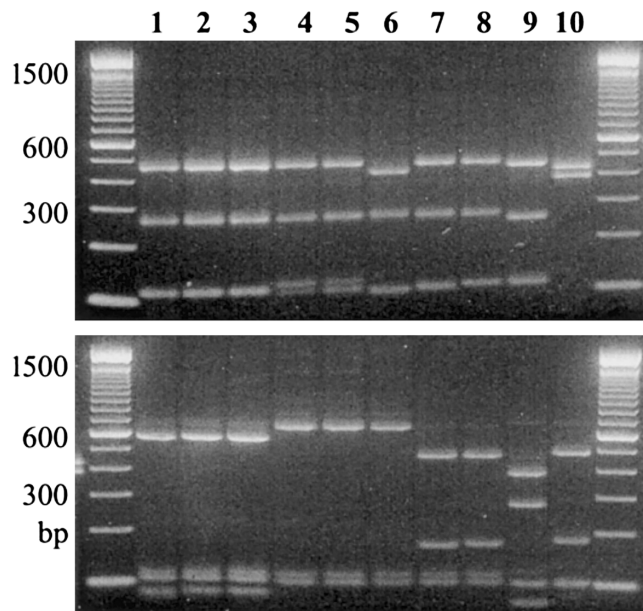


Figure 1. Presence of 5 genotypes of *Cryptosporidium* parasites in children in Lima, Peru, by restriction fragment length polymorphism analysis of small subunit (SSU) rRNA polymerase chain reaction (PCR) products. Nos. on the left are the size of molecular markers, in base pairs (bp). Lanes 1–3, *C. parvum* human genotype; lanes 4 and 5, *C. parvum* bovine genotype; lane 6, *C. parvum* dog genotype; lanes 7 and 8, *C. meleagridis*; lane 9, *C. meleagridis* (PCR product from the heterogeneous copy of the SSU rRNA gene); lane 10, *C. felis*. Top, *SspI* digestion; bottom, *VspI* digestion.

PCR products revealed the presence of 5 genotypes of *Cryptosporidium* parasites (figure 1). Of 85 episodes, 67 (79%) were due to the *C. parvum* human genotype (table 1). In contrast, 18 episodes were due to *Cryptosporidium* parasites that are classically considered zoonotic: 8 were *C. parvum* bovine genotype, 7 were *C. meleagridis*, 2 were *C. parvum* dog genotype, and 1 was *C. felis*. No simultaneous infection with >1 *Cryptosporidium* genotype was detected. For 28 episodes of infection, genotyping results were obtained from multiple (2–5) stool specimens; in all cases, the genotype remained the same throughout the episode. The results of direct sequencing of PCR products of the SSU rRNA and hsp70 genes confirmed the identity of *C. parvum* dog genotype, *C. meleagridis*, and *C. felis* that was obtained by RFLP analysis.

Of the 119 children whose specimens were studied, 13 (11%) had >1 episode of cryptosporidiosis detected during longitudinal follow-up; 2 of the 13 had 3 episodes of infection. The median time between repeated infections in the same person was 10 months (range, 2.1–26 months). Genotype results were obtained from 9 sequential episodes for 4 of these children (figure 2). One child was infected on 2 occasions with *C. parvum* human genotype, once with *C. parvum* dog genotype followed by human genotype, and once with *C. meleagridis* followed by human genotype. One child had 3 episodes of infection geno-

typed: *C. parvum* human genotype, followed by *C. meleagridis* and then by a second infection with *C. parvum* human genotype. Of the 9 episodes presented in figure 2, only 2, the second *C. parvum* human genotype episode for P5444 and the *C. meleagridis* episode for P5266, were associated with diarrhea.

Serum samples were available for HIV testing from 8 children with infection with *Cryptosporidium* parasites of unusual zoonotic genotypes: 6 with *C. meleagridis*, 1 with *C. parvum* dog genotype, and 1 with *C. felis* infection. The serum samples tested had been obtained a median of 14 months after the occurrence of the *Cryptosporidium* infection (range, 8 days to 28 months). All were negative for anti-HIV-1 antibodies.

Characteristics associated with specific *Cryptosporidium* genotypes. Overall, 25 (29%) of 85 episodes of cryptosporidiosis were associated with diarrhea and 19 (28%) of 67 with human genotype, 3 (38%) of 8 with bovine genotype, and 3 (43%) of 7 with *C. meleagridis* infections. None of the small number of infections with *C. parvum* dog genotype or *C. felis* was associated with diarrhea.

No significant difference in age at the time of infection with human genotype versus zoonotic genotypes of *Cryptosporidium* (table 2). Girls were more likely to have zoonotic genotype *Cryptosporidium* parasites than were boys, but the difference did not reach statistical significance. Children with zoonotic genotype *Cryptosporidium* parasites had a somewhat lower mean height-for-age *z* score than did those with human genotype parasites; however, none of the children were severely stunted (defined as height-for-age *z* score of less than –3), and the difference was not significant. None of the children had wasting, as measured by a low weight-for-height *z* score.

There was no significant difference in the percentage of episodes associated with diarrhea or in the duration of diarrhea for infections with human genotype, compared with infections with zoonotic genotypes of *Cryptosporidium*. However, the duration of oocyst detection in stool was significantly longer for infections with *C. parvum* human genotype than for infections with zoonotic genotypes. The mean duration of oocyst shedding was significantly longer for episodes associated with diarrhea (mean, 17.4 days with diarrhea vs. 10.3 days without diarrhea; $P = .03$), and this relationship was true when *C. parvum* human genotype and zoonotic genotypes were examined separately

Table 1. Distribution of 5 *Cryptosporidium* genotypes in 132 stool samples from 80 children in Lima, Peru.

Species, genotype	Children with infection ^a	Episodes of infection	Positive specimens
<i>C. parvum</i>			
Human	65 (81)	67 (79)	111 (84)
Bovine	8 (10)	8 (9)	9 (7)
Dog	2 (2.5)	2 (2)	2 (2)
<i>C. meleagridis</i>	7 (9)	7 (8)	9 (7)
<i>C. felis</i>	1 (1)	1 (1)	1 (1)

NOTE. Data are no. (%).

^a Three children who experienced infections with >1 genotype of *Cryptosporidium* are counted twice (see Results); therefore, percentages total >100%.

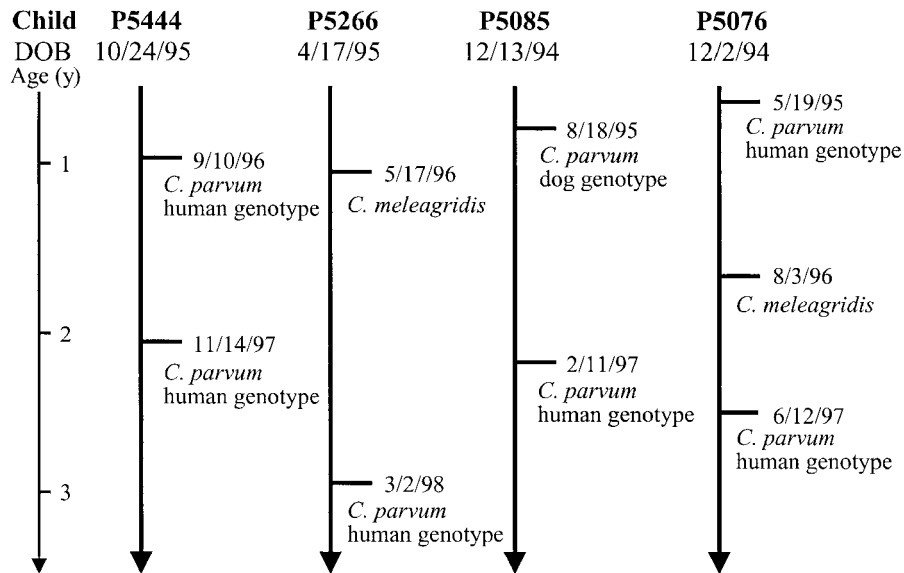


Figure 2. Sequential *Cryptosporidium* infections in 4 Peruvian children (P5444, P5266, P5085, and P5076). DOB, date of birth.

(human genotype: mean, 20.5 vs. 11.3 days; $P = .03$; zoonotic genotypes: mean, 7.5 vs. 5.9 days; $P = .03$). In addition, the mean quantity of oocysts described on slides from stool specimens positive for *C. parvum* human genotype was significantly higher than that for zoonotic *Cryptosporidium* parasites. No difference in the quantity of oocysts was reported for *C. parvum* bovine genotype, compared with that for other zoonotic genotypes ($P = .95$).

For children infected with *C. parvum* human genotype versus those infected with zoonotic genotypes of *Cryptosporidium*, there was no difference in the percentage with piped water, a flush toilet, or chickens, ducks, parrots, rabbits, dogs, cats, guinea pigs, or sheep in their household at the beginning of the study (table 2). There were no cows or calves in this urban community. There were no differences in the socioeconomic status of children with *C. parvum* human genotypes versus those with zoonotic genotypes, as measured by the materials of which the house of the child's family's was constructed.

Discussion

Our data show that, in a setting where cryptosporidiosis is a common endemic childhood infection, novel *Cryptosporidium* species and novel genotypes of *C. parvum* can infect HIV-negative children. In addition to the expected infections with *C. parvum* human and bovine genotypes, some children in our study experienced infection with *C. meleagridis*, *C. felis*, and the *C. parvum* dog genotype. Indeed, in our limited data, cryptosporidiosis due to *C. meleagridis* was almost as common as cryptosporidiosis due to *C. parvum* bovine genotype and, in terms of associated symptoms, was indistinguishable from infection with *C. parvum* human genotype. For the zoonotic

Cryptosporidium parasites, as for the *C. parvum* human genotype, 28%–33% of infections were associated with diarrhea, the duration of diarrhea was >1 week for some children, and children <3 years old were most affected.

Before this study, investigators suggested the possibility that *Cryptosporidium* species other than *C. parvum* could be pathogenic for humans, on the basis of morphologic and biologic similarity, as well as on the genetic relatedness between *C. parvum* and other *Cryptosporidium* spp, such as *C. wrairi*, *C. meleagridis*, and *C. felis* [15, 16]. Infections with *C. parvum* dog genotype, *C. meleagridis*, and *C. felis* were recently reported in patients with AIDS [8, 9]. However, the children infected with novel *Cryptosporidium* genotypes in the present study were HIV negative, were neither severely nor acutely malnourished, and lived under the same conditions of sanitation as other children in the cohort (and millions of other children in developing countries around the world). Our findings suggest that these novel genotypes of *Cryptosporidium* may simply be part of the mix of enteric pathogens in a setting where childhood cryptosporidiosis is endemic.

Nonetheless, our data confirm that the largest percentage of *Cryptosporidium* illness and infection in children in this Peruvian shantytown is caused by the *C. parvum* human genotype. The only clinical characteristics of *C. parvum* human genotype that differed significantly from the zoonotic genotypes were the longer duration of oocyst shedding and higher reported oocyst quantities. A previous study conducted in the United Kingdom reported that oocyst numbers were higher in stool specimens infected with the *C. parvum* human genotype, compared with those infected with the bovine genotype [17]. Our data are consistent with these findings. Adaptation to infect specific hosts is a common phenomenon in *Cryptosporidium* parasites [13,

Table 2. Parameters of *Cryptosporidium* infections, by genotype.

Parameter	Human genotype	Zoonotic genotypes ^a	P
Children			
Age, months ^b	18 (2–97)	15 (8–45)	.55
No. (%) of females ^c	34/62 (55)	13/18 (72)	.19
Height-for-age z score ^{c,d}	−0.94 (−2.6 to 0.8)	−1.17 (−2.6 to 0.73)	.32
Weight-for-age z score ^{c,d}	0.89 (−1.2 to 3.9)	0.84 (−0.4 to 2.5)	.47
No. (%) with flush toilet ^c	22/162 (36)	8/18 (44)	.49
No. (%) with piped water in house ^c	33/62 (53)	11/18 (65)	.40
Infection episode			
No. (%) associated with diarrhea ^c	19/67 (28)	6/18 (33)	.69
Duration of diarrhea, days	4.6 (1–19)	2.8 (1–8)	.43
Duration of oocyst shedding, days	13.9 (1–79)	6.4 (1–31)	.004
No. of oocysts on slide, ^f mean	>1.7	>1.3	.02

NOTE. Data are mean (range) unless otherwise noted. Eighty Peruvian children experienced a total of 85 episodes of infection.

^a Zoonotic genotypes included *C. parvum* bovine genotype (8) and dog genotype (2), *C. meleagridis* (7), and *C. felis* (1).

^b At the beginning of the episode of infection; for 2 children with multiple infections with the *C. parvum* human genotype, only the earlier infection was included in this analysis.

^c Children with *C. parvum* human genotype infections only ($n = 62$) vs. children with any zoonotic infection ($n = 18$).

^d Anthropometric measurements in the month before the infection.

^e Diarrhea during an infection episode defined as having ≥ 1 day with >3 liquid or semiliquid stools and reported by the child's primary caretaker as occurring anytime during the infection, within 1 week before the first stool oocyst detection, or 1 week after the last stool oocyst detection.

^f For 110 specimens genotyped as human vs. 21 specimens genotyped as zoonotic. Intensity of infection was quantified by the number of oocysts counted in a 20- μ L volume of concentrated stool specimen: 1+ (1–50 oocysts), 2+ (51–150 oocysts), and 3+ (>150 oocysts).

15]. It is not surprising that the human genotype *Cryptosporidium* parasites appear to be better adapted to infect humans than do zoonotic *Cryptosporidium* parasites.

Our data are not sufficient to identify the actual routes of transmission of *Cryptosporidium* in this setting. There were no significant differences in animal ownership among families whose children had zoonotic versus human genotype *Cryptosporidium*. However, chickens, ducks, and dogs are common in the study site and are not necessarily confined to the owner's property; environmental contamination by animal feces is widespread. In any case, we cannot say with certainty that the so-called zoonotic types of *Cryptosporidium* were transmitted directly from animal to child, rather than via contamination of water, food, or hands with animal or human feces. There are undoubtedly multiple routes of transmission in this shantytown, and more-focused studies would be required to address this question.

Other characteristics of cryptosporidiosis seen in this study were consistent with cohort data reported previously in the literature. Most *Cryptosporidium* infections detected in this intensely monitored cohort were not associated with diarrhea as in other such studies [18, 19]. However, earlier cohort studies in Peru showed that *Cryptosporidium* infections without diarrhea have a measurable adverse effect on growth [19, 20]; it therefore may be misleading to call nondiarrheal infections "asymptomatic." Although we detected no differences in the number of episodes associated with diarrhea or in the duration

of diarrhea, the significant differences in oocyst numbers and the duration of shedding suggest that the *C. parvum* human genotype may be more likely than zoonotic genotypes to be associated with these more subtle health effects.

As in other cohorts [18, 21], a substantial number of children experienced >1 episode of cryptosporidiosis during the follow-up period. More than half of the repeat infections occurred <1 year after the primary episode of cryptosporidiosis. For the 4 children for whom we were able to genotype repeated infections, the second and third infections were due to homologous *Cryptosporidium* genotypes in some cases and to heterologous genotypes in others. These findings suggest that acquired immunity against *Cryptosporidium* parasites may only be partial or short-lived. Similarly, volunteers experimentally infected with the *C. parvum* bovine genotype were susceptible to secondary challenge with homologous parasites 1 year after primary exposure, although these subsequent infections were not necessarily associated with symptoms [22].

This study extends our knowledge of the heterogeneity of *Cryptosporidium* parasites and will add to the debate concerning the appropriate taxonomy of this genus [7, 15]. It is now clear that not all human cryptosporidiosis is caused by the parasite that we currently call *C. parvum*. Further interdisciplinary work, in which molecular biology is combined with sound epidemiologic analysis, is essential to achieve a better understanding of these important human pathogens.

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