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Serum IgG Responses and Seroconversion Patterns to *Cryptosporidium* gp15 among Children in a Birth Cohort in South India

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The correlates of protective immunity to cryptosporidiosis are not well understood. This study was conducted to assess the effect of maternal serum IgG against *Cryptosporidium* gp15 on responses to this antigen in children with (cases) and without (controls) PCR-confirmed cryptosporidial diarrhea. Maternal sera ($n = 129$) and sera from cases ($n = 39$) and controls ($n = 90$) collected at 3.5, 9, and 24 months of age were tested for serum IgG against *Cryptosporidium* gp15 by enzyme-linked immunosorbent assay (ELISA). Seroconversion patterns were evaluated by estimating probabilities of seroconversion along three time points based on the transition pathways by using a first-order Markov chain process and empirical Bayesian estimates. There was no difference in serum IgG levels or seropositivity rates to gp15 between cases and controls across all time points in children or in IgG levels to this antigen between mothers of cases and controls. The most common transition pathway can be described as a seronegative child at 3.5 months who seroconverts at 9 months and remains seropositive at 24 months. This pattern remained stable irrespective of the serological status of the mother or the case or control status of the child. Children were most likely to be exposed to *Cryptosporidium* for the first time between the ages of 3 and 9 months, and most of the children seroconverted by 24 months. The high degree of seroconversion among control children is suggestive of high rates of asymptomatic transmission in this region.

Cryptosporidium spp. are increasingly being recognized as important diarrheal pathogens worldwide, with the highest associated morbidity and mortality among children in countries where resources are limited (52). Several studies from these countries have documented the highest rates of cryptosporidiosis in children before the age of 2 years (2, 27, 42, 43). The spectrum of symptoms ranges from acute, self-limiting diarrhea in the general population to chronic and life-threatening diarrhea in immunocompromised people (29). Early childhood cryptosporidiosis has been associated with subsequent impairment in growth and physical fitness, as well as cognitive deficiency (12, 20, 34). Transmission can be waterborne, food-borne, or through direct contact with either an infected person or animal (26). Of the 22 species of *Cryptosporidium* identified so far (16), the majority of the human infections are caused by two species—*C. hominis* and *C. parvum* (59).

In India, both hospital- and community-based studies have reported *Cryptosporidium* to be a leading cause of infectious diarrhea in children, with positivity rates (detected by acid-fast staining) in diarrheal stool samples ranging from 1.1 to 18.9% (3). A few studies carried out by us (1, 2) and others (15, 17, 35) using molecular techniques have found *C. hominis* to be the predominant species affecting the Indian children.

Previously, we showed that *Cryptosporidium* was the most common cause of parasitic diarrhea in children, in both hospital and community settings (1, 2) in Vellore in south India. In a longitudinal study of children with cryptosporidial diarrhea from a community-based birth cohort, we found that 40% of children had multiple infections (5).

Both cell-mediated and antibody responses are thought to be involved in protection from and resolution of cryptosporidiosis (10, 48). However, although there have been several studies on immune responses following cryptosporidiosis, the correlates of protective immunity are not well understood (reviewed in reference 10). Previous longitudinal and population-based cross-sectional studies have demonstrated that the prevalence and magnitude of the serum IgG response to specific cryptosporidial antigens increase with increasing age and infection experience (14, 45). A negative correlation between age and serum IgM positivity has also been shown, suggesting a higher susceptibility to cryptosporidiosis in younger subjects (30).

Most studies on antibody responses have used *C. parvum* lysates as an antigen for enzyme-linked immunosorbent assays (ELISAs) (27, 38, 50, 55, 56). However, more recent studies have employed native or recombinant forms of the 15- to 17-kDa surface glycoprotein gp15 (4, 6, 32, 45), one of the most immunodominant antigens (10), which we and others cloned and characterized (13, 46, 54, 58). In a case-control study of cryptosporidiosis in Bangladeshi children with diarrhea, there was a significant increase in serum IgG responses to gp15 over 3 weeks of follow up in cases with cryptosporidiosis compared to controls with diarrhea but no *Cryptosporidium* infection (6). Recently, we showed that there was significant increase in serum IgG levels to gp15 following the first episode of cryptosporidial diarrhea in children

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in the birth cohort study in Vellore, and the increase in seropositivity had a nonlinear pattern, with a peak response between 8 and 11 weeks postexposure (4, 51). The presence of preexisting serum antibodies to gp15 has been associated with protection from diarrheal symptoms in naturally or experimentally infected adults (reviewed in references 10 and 48). This antigen also induces cellular immune responses in previously infected individuals (44). Based on its immunogenicity, protective efficacy in animal models, and ability to induce antibody and cell-mediated responses in humans, gp15 has been considered a putative vaccine candidate (10, 44).

The effect of maternal serum antibodies or breastfeeding on acquisition of cryptosporidial infection is not clear. In a prospective cohort study in Lima, Peru, no difference was found in the prevalence of cryptosporidiosis among breastfed children of mothers with high, medium, or low antibody titers (53). Also, while some studies have found breastfeeding to be protective (9, 25), other studies have either failed to find an association (21, 27, 37) or have found breastfeeding to be a risk factor for acquisition of cryptosporidiosis (7, 36).

This study was conducted to assess the effect of maternal serum antibodies to gp15 on serum IgG responses to this antigen longitudinally from birth to 2 years of age in children with and without cryptosporidial diarrhea in the birth cohort study in Vellore and to evaluate the patterns of seroconversion in these children.

MATERIALS AND METHODS

Study subjects and follow-up. The subjects in this study were part of a birth cohort of 452 children recruited for a study on the natural history of rotavirus from four adjacent semiurban slums in Vellore, south India. The details of study enrollment and follow-up have been reported previously (18). The study was approved by the Institutional Review Board of Christian Medical College, Vellore, and written informed consent was obtained from the parents. Data on sociodemographic, environmental, and clinical characteristics were collected during the study. Each child in the cohort was visited at home twice a week to record any morbidity or diarrhea during the period since the last visit. Monthly anthropometric (height and weight) measurements were also obtained from the study child. Stool samples were collected fortnightly or whenever the child had an episode of diarrhea, and the child was followed up daily until cessation of the diarrheal episode. Diarrheal stool samples obtained from the child were tested to identify bacterial, parasitic, or viral agents causing diarrhea, and the results obtained were reported previously (1, 8). All diarrheal samples were screened for *Cryptosporidium* spp. by microscopic examination of acid-fast-stained smears followed by PCR at the 18S rRNA locus and restriction fragment length polymorphism (RFLP) for species identification (1).

Serum samples. A maternal serum sample was obtained either at the time of delivery or soon after recruitment (if the delivery was not institutionalized). Serum samples from the children in the cohort were collected every 3 months during the first 2 years of life and every 6 months during the third year. All serum samples were stored in aliquots at −20°C.

Selection of cases and controls. A total of 58 cryptosporidial diarrheal episodes (defined as an episode of diarrhea with detection of *Cryptosporidium* spp. by acid-fast microscopy of the stool sample) were observed in 53 children in the cohort, the majority of which were identified as *C. hominis* (1). Of these, 42 children experienced one or more episodes of cryptosporidial diarrhea at or below the age of 2 years. Details on collection of stool samples and results from detection and molecular typing of *Cryptosporidium* spp. in diarrheal stools (1) and serum IgG responses to gp15 following an episode of cryptosporidial diarrhea (4) have been published previously.

Ninety-three children who did not experience any episode of cryptosporidial diarrhea up to 2 years of age were identified by screening all diarrheal stool samples by PCR from 124 randomly selected children in the cohort.

Maternal sera collected at delivery and sera from children at 3.5, 9, and 24 months of age were available for 39 of 42 children with cryptosporidial diarrhea (cases) and 90 of 93 children with no cryptosporidial diarrhea (controls) at or below the age of 2 years. Hence, we restricted the study to 129 children (39 cases and 90 controls) for whom the complete set of serum samples (maternal as well as child sera at all the time points) was available.

Anthropometric measurements. The height-for-age (HAZ), weight-for-height (WHZ), and weight-for-age (WAZ) *z*-scores were calculated using the 2006 WHO child growth standards as the reference population (57). Children were then classified as stunted (HAZ < -2 standard deviations [SD]), wasted (WHZ < -2 SD), underweight (WAZ < -2 SD), or normal based on their *z*-scores.

ELISA. All serum samples were tested for IgG levels to *C. parvum* gp15 by ELISA as described previously (4, 6). The results were expressed in ELISA units (EU).

Statistical analysis. Data were analyzed using STATA 10.0 for Windows (StataCorp, College Station, TX) software. A particular sample was considered to be seropositive if the IgG level to gp15 (expressed in EU) was >20 EU and seronegative if it was ≤ 20 EU. The serum IgG levels to gp15 were log transformed, and to avoid exclusion of samples with no detectible antibody following log transformation, a relatively small value of "1" was added to the original IgG levels prior to transformation. The proportion of seropositive samples and the difference in IgG levels (for the seropositive samples) at different time points were compared between cases and controls. Similarly, the differences in baseline sociodemographic and hygiene variables and the number of diarrheal episodes between cases and controls were analyzed. The prevalences of stunted, wasted, and undernourished children at each of the three time points were also compared between cases and controls. The χ^2 test or Fisher's exact test (as applicable) was used to compare the categorical variables, and the *t* test or Mann-Whitney U test (as applicable) was used to compare the continuous variables.

A child was considered to have seroconversion at a particular time point when the corresponding serum sample was positive, but the preceding serum sample was negative. At a given age, the ratio of the number of seronegative to seropositive children (N/P ratio) was estimated, and the time point at which majority of the children seroconverted was ascertained.

Similar to the N/P ratio, a transitional probability of having a particular serological status at a certain time point, or a node, was estimated by calculating the ratio of the number of individuals at a particular node to the number of individuals at a previous node, thereby considering it as a first-order Markov chain process (49). In order to determine the most prevalent transition pathway in the study population, a joint probability was computed by multiplying the probabilities at a particular transition node by that of the previous node. The pathway with the highest joint probability at the end node was considered to be the most common, or dominant.

To assess the uncertainties associated with assigning of seropositive status and a finite sample size, we provide empirical Bayesian estimates for the mean and standard deviation of counts and their relative frequencies, which were obtained using Monte Carlo simulations based on 1,000 cycles. Each simulated cycle imitates the sequential process for three time points and uses empirical estimates of probability of successes embedded in a binomial distribution. Thus, the estimate for the terminal nodes represents the joint probability of two previous stages.

RESULTS

Sociodemographic and other characteristics. Comparison of the sociodemographic status, standards of hygiene, duration of exclusive and partial breastfeeding, birth weight, and presence of

TABLE 1 Proportions of children who seroconverted over time and mean anti-gp15 IgG antibody levels among the seropositive children at the three time points measured

Time (mo)	Result for: Cases (n = 39) No. (%) of children positive	Cases (n = 39) Mean (SD) gp15 EU[a]	Controls (n = 90) No. (%) of children positive	Controls (n = 90) Mean (SD) gp15 EU[a]	P value[b]
3.5	9 (23.1)	3.5 (0.4)	32 (35.6)	3.9 (0.5)	0.207
9	28 (71.8)	4.1 (0.7)	71 (78.9)	3.9 (0.6)	0.099
24	37 (94.9)	4.1 (0.1)	85 (94.4)	4.2 (0.6)	0.221

[a] ln (antibody level + 1) among the seropositive samples.
[b] P value by t test comparing the mean antibody levels among the seropositive cases and controls.

animals in the household showed no statistically significant differences between cases and controls (see Table S1 in the supplemental material). Also, no significant differences in the numbers of children who were stunted, wasted, or underweight were observed between cases and controls at any of the time points (see Table S2 in the supplemental material).

Maternal and longitudinal serum IgG levels to gp15 in cases and controls. Of a total of 129 maternal serum samples analyzed, 115 (89.2%) were seropositive. However, only 41 (31.8%) of the 129 children were seropositive at the age of 3.5 months. This increased to 99 (76.7%) at 9 months, and by 24 months, almost all children (122, [94.6%]) were seropositive. When split into cases and controls, the proportions of seropositive mothers were similar across both groups (32 [82.1%] for cases versus 83 [92.2%] for controls; $P = 0.088$). Similarly, the proportions of seropositive children were comparable between cases and controls for 3.5 months (9 [23.1%] for cases versus 32 [35.6%] for controls; $P = 0.162$), 9 months (28 [71.8%] for cases versus 71 [78.9%] for controls; $P = 0.381$), and 24 months (37 [94.9%] for cases versus 85 [94.4%] for controls; $P = 0.922$).

When the serum IgG levels to gp15 (log transformed) were compared among the seropositive mothers, no difference in the antibody levels was noticed between the mothers of cases (mean, 4.1; SD, 0.5) and controls (mean, 4.1; SD, 0.4) ($P = 0.744$]). Similarly, the IgG levels were comparable between the seropositive cases and controls at 3.5 ($P = 0.207$), 9 ($P = 0.099$), and 24 ($P = 0.221$) months (Table 1).

Seroconversion patterns in cases and controls. In order to ascertain the time point at which the majority of children seroconverted, the N/P ratios at different time points were calculated. When analyzed for all children in the study irrespective of their case-control status (see Fig. S1a in the supplemental material), it was found that a majority of children (88 [68.2%]) were seronegative at age 3.5 months (N/P ratio, 2.15), many of whom either remained seropositive or seroconverted (99 [76.7%]) by 9 months (N/P ratio, 0.30). Almost all children became seropositive by 24 months of age (122 [94.7%]; N/P ratio, 0.06). The seroconversion patterns were similar for cases (see Fig. S1b in supplemental material) and controls (see Fig. S1c in supplemental material), with the majority of the children becoming seropositive at 9 months (N/P ratios of 0.39 for cases and 0.27 for controls).

Association of maternal IgG levels with seroconversion status. The transition probabilities between seropositive and seronegative status were calculated for children of seropositive (see Fig. S1d in the supplemental material) and seronegative mothers (see Fig. S1e in supplemental material) separately. The commonest transition pathway was a seronegative child at 3.5 months who seroconverted at 9 months and remained seropositive at 24 months (0.52). This remained stable irrespective of the serological status of the mothers (0.50 for children of seropositive mothers versus 0.65 for children of seronegative mothers) or the case-control status (0.56 for cases versus 0.50 for controls).

Seronegative mothers were more likely to have seronegative children: at 3.5 months, only 14.3% of children of seronegative mothers were seropositive compared to 33.9% seropositive children of seropositive mothers, although this difference was not statistically significant ($P = 0.223$). The difference, although small, remained at 9 months (71.4% versus 77.4%; $P = 0.738$) and 24 months (85.7% versus 95.7%; $P = 0.168$) (Fig. 1A). When split by the case-control status of the child (Fig. 1B and C), the difference was statistically significant in control children at 24 months (71.4% versus 96.4%; $P = 0.047$).

Bayesian estimation of the probability of seroconversion. The empirical Bayesian estimates for the mean and standard deviation of counts and relative frequencies for each node obtained from the simulations closely resembled the sample estimates (see Table S3 in the supplemental material). For example, the probability of a child remaining seronegative at 3.5 months, seroconverting at 9 months, and thereafter remaining seropositive at 24 months, the most common pathway, was observed to be 0.52 from the sample, whereas the mean of the simulated probabilities calculated from the Bayesian estimation was 0.52 (SD, 0.04). Similarly, the probabilities of a child being seropositive at 3.5 months and remaining so at 9 and 24 months were estimated to be 0.24 from the sample and 0.24 (SD, 0.04) from the simulations.

DISCUSSION

Seroepidemiological studies of cryptosporidiosis, particularly in areas of endemicity where resources are limited, have consistently documented higher prevalence of infection than those reported from parasitological studies (24, 30, 33, 56). In our study, almost all children (94.6%) became seropositive by the time they attained the age of 2 years. Similar findings have been reported by Zu et al. (60) in a study in which the vast majority of children residing in an impoverished area in Fortaleza, Brazil, were reported to have acquired IgG antibodies to *Cryptosporidium* by 2 years of age. The children in our study resided in an urban slum area (18) with high levels of drinking water contamination (11). Previous studies have also reported that the seroprevalence of cryptosporidiosis was higher among people residing in areas with poor quality drinking water and inadequate sanitation facilities (28, 55).

The rising seroprevalence with increasing age of the child suggests an increased level of exposure and reexposure to the organism at the subclinical level. In a study of Peruvian children, the magnitude of antibody response was found to be related to the age of the child and the number of previous infections (45). The risk of exposure to *Cryptosporidium* spp. was lowest in children up to the age of 3 months, possibly due to passive protection from breast milk antibodies, lower environmental exposure, or both. In other studies, too, it has been noticed that seropositivity to cryptosporidial antigens was lowest among children aged 4 to 6 months, increasing steadily thereafter (14, 50).

In our study, the seroconversion patterns were similar in cases

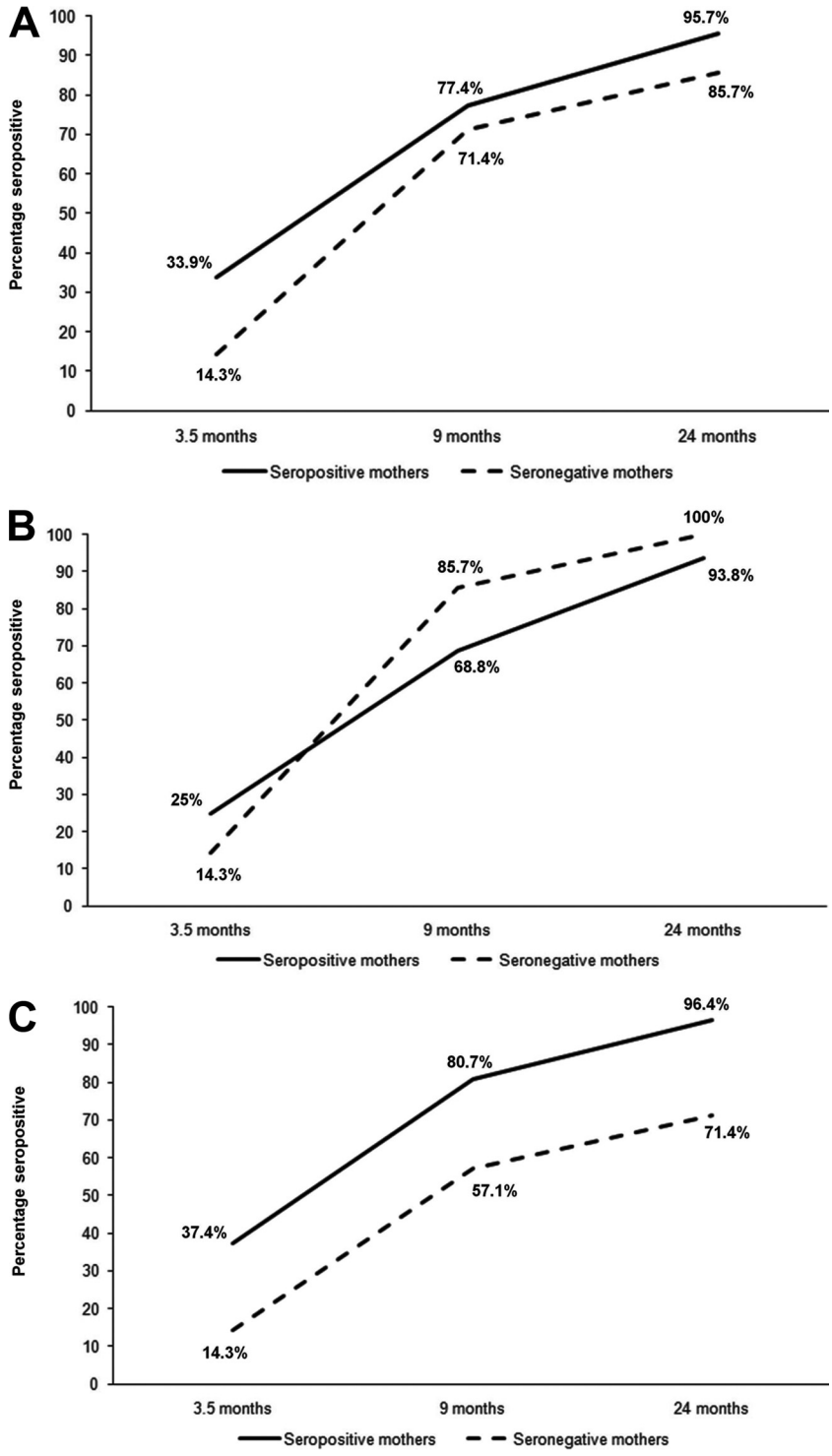

FIG 1 Proportion of seropositive children across the three time points (3.5, 9, and 24 months) stratified by the serological status of the mother in all children (n = 129) (A), in children with PCR-confirmed cryptosporidial diarrhea (cases) (n = 39) (B), and in controls (n = 90) (C). The percentage of children who are seropositive at each time point is mentioned.

and controls across all time points. In a prospective study of Israeli Bedouin children, no difference in the patterns and magnitudes of serum antibody response in infants with and without cryptosporidiosis was noticed (50). Similarly, almost all children residing in a Thai orphanage had detectible levels of *Cryptosporidium*-specific IgG antibodies, irrespective of their infection status (24). These findings are indicative of the high degree of cryptosporidial infection among children residing in resource-poor settings.

In this study, a majority of children were observed to have seroconverted by 9 months of age, irrespective of the serological status of their mother or their case-control status. This suggests that, in an area of high endemicity, a child is more likely to first be exposed to *Cryptosporidium* spp. between the ages of 3 and 9 months. This has important implications for the timing of administration of the first dose of human cryptosporidial vaccine, when available. Vaccines are most effective when the average age of vaccination is lower than the average age of first infection (39).

Typically, studies on the serological response to cryptosporidiosis are cross-sectional in nature (14, 32, 56, 60) or consist of "pre/post" study designs (4, 6, 40, 45) and present seroconversion as a proportion of positive tests. In longitudinal studies, multiple pairwise comparisons can be cumbersome and have the potential to reduce statistical power. Previous studies have demonstrated that the IgG response to *Cryptosporidium* infection is short-lived (4, 19, 47) and thus requires novel analytical tools to track reversible changes in longitudinal data. The proposed statistical approach to analyze the temporal patterns of serum IgG levels in response to gp15 in children with and without cryptosporidial diarrhea is based on Markov chain modeling, which allows us to examine the probability of the occurrence of all possible transition pathways and ascertain the most common one. Also, the use of the empirical Bayesian method to estimate the degree of uncertainty associated with the observed seroconversion patterns offers a reliable alternative to that solely based on direct comparison of seroconversion rates at specific time points as it utilizes all available information on the sequence of successful outcomes (in our case, a seroconversion), thereby minimizing the measurement error. The proposed approach of estimation transitional probabilities coupled with graphical presentations allows for a comprehensive presentation of longitudinal data.

A major limitation of this study was that the surveillance stool samples were not screened for the presence of asymptomatic cryptosporidial infections. Several studies, including our own, conducted in settings where resources are limited have documented high rates of asymptomatic transmission of *Cryptosporidium* spp. in children (22, 23, 37, 41, 50). In a study of sequential cryptosporidial infections among a subset of children in the same cohort, asymptomatic shedding of *Cryptosporidium* oocysts was noticed in a small proportion of children (5). Asymptomatic cryptosporidiosis among children in the control group could have increased the seropositivity rates in that group, which might explain the observed lack of difference between the cases and controls.

An important assumption of this study was that the rate of antibody transfer was uniform for all children (i.e., children with high maternal antibody levels had higher antibody levels at birth). Although studies examining pairs of maternal and cord blood samples have found good correlation in the antibody levels between them (14, 50, 60), the transfer of antibodies could be influenced by factors like prematurity and gestational age (31). Data on cord blood samples might provide more robust estimates. Another limitation of our study was that the transfer of protective immunity conferred by maternal IgA (breast milk in the mother and fecal samples in the children) was not explored.

In conclusion, the results of this study suggest very high levels of cryptosporidial transmission among children residing in a semiurban slum area in south India. The risk of infection was lowest at the age of 3 months, irrespective of maternal immune status, thereby suggesting a lower risk of exposure or passive protection from breast milk antibodies among newborns. The high degree of seroconversion in children without any history of cryptosporidial diarrhea is also suggestive of high rates of asymptomatic transmission among children residing in this area. Further studies are required to quantify the burden of asymptomatic disease and the degree and duration of serological responses in children with multiple symptomatic and asymptomatic cryptosporidial infections in this community.

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