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# *Giardia duodenalis* Assemblages Associated with Diarrhea in Children in South India Identified by PCR-RFLP

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## Abstract

Giardial diarrhea in a birth cohort of 452 children in an urban slum in South India was characterized. Of the 155 episodes that occurred in 99 children, 73% were acute diarrhea. Children with better educated mothers and a toilet at home had lower odds of acquiring giardial diarrhea, whereas low socioeconomic status and drinking municipal water were associated with greater risk. Children with co-infections tended to have a slightly longer duration of diarrhea ($P = 0.061$) and showed significantly more wasting after an episode than children with diarrhea resulting from *Giardia* alone ($P = 0.032$). Among the 99 cases, 50 diarrheal and 51 asymptomatic *Giardia* positive samples were genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) at the triose phosphate isomerase gene. Assemblage B was predominant both in giardial diarrhea (80%) and asymptomatic giardiasis (94%). Children with Assemblage A subgroup-II alone or dual infections with both assemblage A and B had diarrhea more frequently ($P = 0.07$).

*Giardia duodenalis*, a common intestinal protozoan parasite causes infections that range from asymptomatic cyst passage and acute diarrhea to a syndrome of chronic diarrhea, weight loss, and malabsorption.[1] In children in developing countries, giardiasis is associated with stunting and malnutrition and documented to have adverse effects on success at school and cognitive function.[2] Molecular characterization of *Giardia* from humans and animals has been carried out at several loci, including glutamate dehydrogenase (*gdh*),[3] β-giardin,[4] small subunit ribosomal RNA (*SSU rRNA*),[5] and the triosephosphate isomerase (*tpi* or *tim*)[6] genes. These studies have shown that the two major genotypes or assemblages causing human infections are assemblages A and B. In India, reports on the prevalence of giardiasis in children range from 2.6[7] to 32%.[8] In this study, we aimed to describe endemic giardiasis in a birth cohort of children from an urban slum community in Vellore, South India, and identify the prevalent giardial assemblages in the community.

The 452 children in the birth cohort[9] were originally recruited for studies on rotaviral[10,11] and cryptosporidial diarrhea[12] and were followed up twice-weekly up to the age of 3 years. The study setting comprised of three adjacent urban slums in Vellore with an area of around 2.2 km$^2$ and population of ~33,390. Children with diarrhea were assessed clinically, and

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details of the number of stools passed per day, any associated fever or vomiting, and treatment given was recorded daily until the cessation of diarrhea. Demographic details were recorded at the start of the study. Each stool sample was given a unique identification number and stored in aliquots at −70°C and 4°C. All diarrheal stool samples were screened for enteric parasites, including *Giardia* cysts and trophozoites by direct wet mount microscopic examination of fresh stool samples. In addition, modified acid fast staining was carried out to identify *Cryptosporidium* spp. Bacterial enteric pathogens, including *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Aeromonas* spp., and enteropathogenic *E.coli*, were identified by stool culture on blood agar and selective media, including sorbitol MacConkey agar, xylose lysine desoxycholate agar, and thiosulphate citrate bile salt sucrose agar. Rotavirus was screened for by enzyme-linked immunosorbent assay (ELISA) (Dako IDEIA, UK) and sapovirus by a hemi-nested polymerase chain reaction (PCR).[13] Data generated during this study were double entered using Epi Info 2000 software (CDC, Atlanta, GA) and analyzed using STATA version 9.0 (Stata Corp., College Station, TX). Statistical comparisons were made using Fisher's exact and $\chi^2$ tests for categoric variables and Wilcoxon signed-rank test for continuous variables. A univariate logistic regression was carried out for risk factor analysis and odds ratios (OR) (with 95% confidence interval [CI]) calculated.

For this study, an episode of giardial diarrhea was defined as at least 1 day of diarrhea (three loose stools in a 24 hour period) followed by at least 2 days without diarrhea.[14] Acute diarrhea was defined as fewer than 4 days and persistent diarrhea as more than 14 days. Children with diarrhea more than 3 days but fewer than 14 were described as indeterminate. A relapse of giardial diarrhea was defined as a second episode commencing between 2 and 7 days after the conclusion of the original *Giardia* diarrhea, and a recurrence was defined as infection occurring more than 7 days after the initial *Giardia* diarrhea.[14] The study was approved by the Christian Medical College, Vellore Institutional Review Board, and informed consent was obtained from the parents of these children.

During the follow-up, almost 2,000 diarrheal episodes were studied between January 2002 and April 2006. There were 155 episodes of giardial diarrhea in 99 children, 53 of whom were male. The mean (SD) age at first episode was 19.34 (7.89) months. The characteristics of giardial diarrhea among children in this slum community have been described in Table 1. Although no child had persistent diarrhea, 1 child had diarrhea for 9 days and 5 children had diarrhea for 8 days. Clinical features, severity of diarrhea, and anthropometric measurements taken after the episode were compared between only *Giardia*-associated episodes and 36 episodes of *Giardia* co-infection with other enteric pathogens, including rotavirus (25%), sapovirus (11.1%), *Cryptosporidium* spp. (16.7%), *Vibrio cholerae* (11.1%), *Shigella* spp. (8.3%), *Salmonella* spp. (8.3%), enteropathogenic *E. coli* (2.8%), and *Aeromonas* spp. (30.6%) (Table 2). The World Health Organization (WHO) growth reference curves were used to interpret anthropometric data and calculate Z-scores.[15] To identify the risk factors associated with acquiring giardial diarrhea, we also compared sociodemographic data between 99 children with at least one episode of giardial diarrhea with the remaining 353 children in the birth cohort. Although sex, low birth weight, maternal age at birth, family size, number of siblings, and presence of domestic animals or pets in the household were not associated with an increased OR of giardial diarrhea, low socioeconomic status (OR, 95% CI) (1.62, 1.00–2.63) and drinking municipal water (1.69, 0.20–14.24) were associated with giardial diarrhea. Better maternal education (0.57, 0.33–1.01 for grades 1–5 and 0.31, 0.15–0.67 for grades 9 and above) and presence of a toilet at home (0.61, 0.35–1.08) were found to be associated with protection from giardial diarrhea, whereas education of the head of the household, duration of breast feeding, and hand washing were not protective.

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To identify the prevalent assemblages in the community, DNA was extracted from 50 randomly selected cases with giardial diarrhea with the QIAamp stool DNA minikit (Qiagen Inc., Valencia, CA). Previously published primers and protocols were used for detection of assemblages A and B at the *tpi* locus by a nested PCR[6] with a minor modification of adding 5% DMSO to the PCR reaction as described by other workers for this GC-rich gene.[16] The nested *tpi* PCR showed a 100% correlation with microscopy. This was followed by restriction fragment length polymorphism (RFLP) with *RsaI* to identify assemblage A subgroups (Figure 1). As suggested by other workers,[3] the PCR-RFLP was validated by sequence analysis of one fecal sample classified as assemblage A. There was > 98% sequence identity with the Genbank accession number U57897, assemblage A group II.[17] We also included as controls, 51 children who had asymptomatic giardial infections diagnosed by stool microscopy. These children lived in the same urban slum area as the children with giardial diarrhea, but only 5 belonged to the birth cohort. The remaining 46 samples were collected from among children attending the primary health center in the study area for illnesses not related to the gastrointestinal tract between March and July 2008. Informed consent was obtained from the parent.

A majority of infections among the diarrheal and asymptomatic samples genotyped were a result of assemblage B (80% and 94%), corresponding to the findings of previous studies in the region, including India,[16] Bangladesh,[18] Philippines,[19] and also from Europe[3,20] and Brazil.[21] Among the remaining diarrheal samples, 5 were assemblage A and by RFLP analysis, belonged to subgroup II, an anthroponotic subgroup and 5 had a dual infection with assemblages A and B as previously reported elsewhere.[6] Among the dual infections, only one assemblage A isolate could be genotyped by RFLP, and this also belonged to group II. In the asymptomatic children, only 2/51 children were infected with assemblage A-II and 1 with both assemblages B- and A-II. When both single and dual infections were considered, children with Assemblage A infection ($N = 13$) tended to have diarrhea more frequently ($N = 10$) than not ($N = 3$) ($P = 0.074$), but the association was not as strong as demonstrated by Haque and others.[18] There was no difference in features of diarrhea like severity (measured as maximum number of stools per day), dehydration, duration or malnutrition after diarrhea or associated symptoms, such as vomiting and fever between children with assemblage A and B infection. Previous reports on association of a particular genotype with diarrhea have been conflicting, with some reports suggesting an increased odds of diarrhea for assemblage A,[18,22–24] whereas others have shown a correlation of symptoms,[25,26] increased oocyst shedding,[21] and persistence of infection with assemblage B.[25] Two previous studies have however, reported no association of genotype with symptoms.[21,27]

This pilot study provides information on the characteristics of endemic giardial diarrhea in children in the community in South India, and is the first to document the common assemblages causing symptomatic and asymptomatic infections in the community. The prevalence of assemblage B and the occurrence of only subgroup A II indicate an anthroponotic transmission cycle as has been seen in other countries in Asia[19] and the Indian subcontinent.[18] However, the role of domestic animals and livestock as a potential source of infection for humans in the community also needs to be researched. Larger studies with asymptomatic and symptomatic cases followed up longitudinally along with glutamate dehydrogenase (*gdh*) gene analysis to subtype assemblage B isolates could lead to better knowledge of the association of these assemblages with diarrhea in children.

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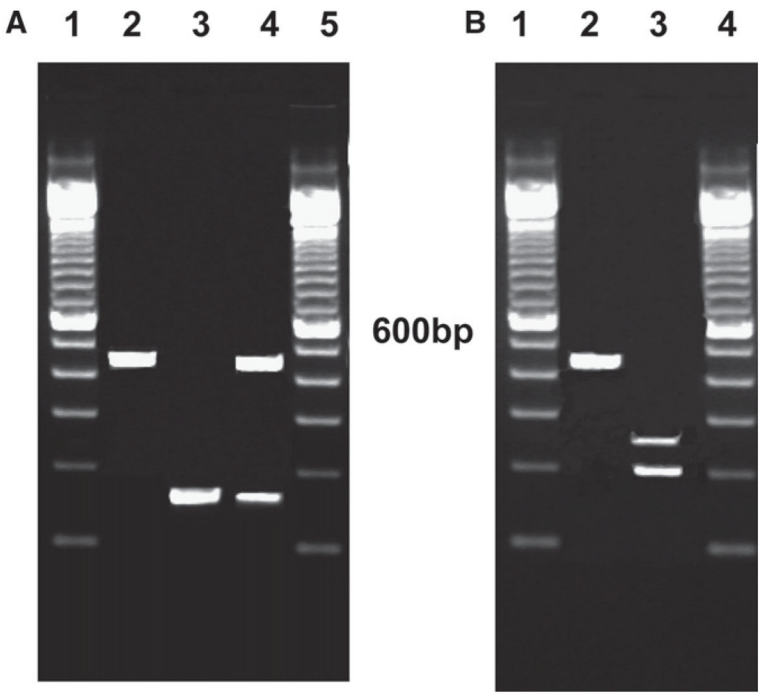


**Figure 1.**

**A**, Nested PCR at the **tpi** locus for identification of *Giardia* assemblages (Lane 1 and 5—molecular weight marker; Lane 2—assemblage A; Lane 3—assemblage B; Lane 4—coinfection of assemblage A and B). **B**, **TpiA** RFLP with **RsaI** for assemblage A (Lane 1 and 4—molecular weight marker; Lane 2—assemblage A–I; Lane 3—assemblage A–II).

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## Table 1

Characteristics of Giardial diarrhea among children in a community birth cohort in South India

| **Features of Giardial diarrhea** | ***N* = 155 episodes** |
|---|---|
| Acute (< 4 days) | 113 (72.9%) |
| Indeterminate (4–14 days) | 42 (27.1%) |
| Persistent (> 14 days) | 0 |
| Average duration of diarrhea (IQR) | 2 days (2–4 days) |
| Maximum duration of diarrhea | 9 days |
| | *N* = 99 cases |
| Multiple episodes | 34 cases |
| Maximum number of episodes | 5 episodes |
| Relapses | 12 (7.7%) |
| Recurrences | 44 (28.4%) |
| Median interval between recurrent episodes (IQR) | 125 days (58–167 days) |

IQR = interquartile range.

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## Table 2

Comparison of clinical features in children with *Giardia* diarrhea with children co-infected *Giardia* and other enteric pathogens

| | ***Giardia* with coinfection *N* = 36** | ***Giardia* alone *N* = 119** | ***P* value*** |
|---|---|---|---|
| Median duration of diarrhea (IQR) | 3 (2–4) | 2 (1–3) | 0.061 |
| Acute diarrheal episodes | 22 | 91 | 0.069 |
| Severity of diarrhea (maximum number of stools/day) | | | |
| 1–3 episodes/day | 8 | 19 | |
| 4–5 episodes/day | 6 | 24 | 0.645 |
| ≥ 6 episodes/day | 20 | 74 | |
| Malnutrition† | | | |
| Stunted | 26 | 70 | 0.173 |
| Wasted | 12 | 19 | 0.032 |
| Underweight | 20 | 49 | 0.180 |
| Associated fever‡ | 6 | 15 | 0.573 |
| Associated vomiting‡ | 4 | 14 | 1.000 |

*Fisher's exact test was used for all analysis, except comparison of the median duration of diarrhea for which the Wilcoxon signed-rank test was used.

†Children with height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height (WHZ) scores less than −2 were categorized as stunted, underweight, and wasted, respectively.

‡Clinical data not available for 2 children with co-infection and for 2 children without co-infection.

IQR = interquartile range.