Original Article

Detection and Genetic Characterization of *Giardia intestinalis* in Children with Gastrointestinal Symptoms by PCR RFLP in Sikkim, India

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Abstract

Background: This study is aimed to detect *Giardia* and genetically characterize *Giardia intestinalis* among children in Sikkim, India. **Materials and Methods:** A total of 400 stool samples were collected from the children (<15 years) with gastrointestinal symptoms, who attended Central Referral Hospital and Sir Thodup Namgyal Memorial Hospital. *Giardia* cysts were detected by microscopy from the stool samples and were genetically characterized by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) targeting glutamate dehydrogenase gene (*gdh*). **Results:** *Giardia* cysts were detected in 20/400 (5% incidence rate). Out of 20 samples, 13 (65%) were successfully assayed by PCR-RFLP. The PCR product of *gdh* gene was digested by *BsPL1* and *RsaI* and isolated 53.7% of BIV, 38.5% of AII and B mixed, and 7.8% of BIII assemblages. There was no AI assemblage found in this study. Diarrhea and abdominal pain were the common complaints associated with giardiasis. **Conclusions:** PCR-RFLP targeting *gdh* gene locus is a reliable, easy, and cost-effective method to identify *G. intestinalis* and its assemblages. This is the first report on the prevalence and genetic variability of human giardiasis in symptomatic children in Sikkim, India.

Keywords: Children, gastrointestinal, genotyping, *Giardia intestinalis*, Sikkim

Introduction

Giardia intestinalis (synonyms *Giardia lamblia* and *Giardia duodenalis*) is one of the most common intestinal parasites which affects children worldwide.[1,2] It causes giardiasis which results in malnutrition, stunted growth, and cognitive impairment.[3] Giardiasis in human is mostly transmitted through ingestion of cysts contaminated with food and water.[1] The genus *Giardia* belongs to phylum Sarcomastigophora, class Zoomastigophora, and a member of the order Diplomonadida.[4] Eight assemblages (A-H) have been described, of which all human isolates are characterized into either assemblage A or B.[5] Assemblage AI is also detected in livestock, cats, and dogs, whereas assemblage AII is exclusively in humans.[6] Approximately 200 million people have symptomatic giardiasis in Asia, Africa, and Latin America and about 500,000 new cases are reported each year among children.[5] Till date, the genotyping studies of *G. intestinalis* are relied on using *β-giardin*, *gdh*, *Tpi*, SSU, RNA, *efla,* and variant surface protein (*vsp*) genes from different hosts.[3,7] Distribution of the human-associated assemblage varies geographically, and the use of primers based on *Tpi* marker detected more mixed infection with assemblage A and B than other primers.[8,9] The study is aimed to identify the *G. intestinalis* assemblage and subassemblage in children with gastrointestinal symptoms in Sikkim, India.

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Materials and Methods

This prospective study was conducted using stool samples from 400 children attending Sir Thodup Namgyal Memorial Hospital and Central Referral Hospital located in East Sikkim, during April 2015–April 2017. Children in the age group of 0–15 years, who

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presented with gastrointestinal symptoms (diarrhea, vomiting, nausea, weight loss, fatigue, and bloating), were investigated for intestinal parasitic infection before administration of medication. This study was approved by the University Ethical Committee. Written informed consent was obtained from parents/guardian.

Stool sample collection and processing

The children/guardians were given a labeled, leak-proof container with a plastic scoop (HiMedia) to collect sample as per the standard procedure of the WHO and processed within 4 h of collection. The samples were examined microscopically after formal ether concentration technique using saline and iodine mount coverslip preparations.

DNA extraction

DNA was extracted directly from the specimen using commercial kit *Helini* pure fast stool DNA Minispin Prep Kit (Catalog no. 2006; *Helini biomolecules*, Chennai, India) using the manufacturer's instruction. To enhance the recovery of DNA, before adding the lysis buffer and proteinase K, the suspension of the stool-processing buffer and the stool were subjected to freezing and thawing process (−80/+80) with added glass beads (HiMedia) for 10 min alternatively for 1 h and the final elution was made at 50 µl and stored at −20°C.

Polymerase chain reaction amplification

The amplification of *gdh* was performed by a semi-nested polymerase chain reaction (PCR) to get the fragment of approximately 432 bp. The primers used in the first PCR reaction were GDHeF, GDHiR, and GDHiF, and GDHiR was used in second PCR reaction as previously described.[7] PCR reaction mixtures consisted of 10 µl of Red Dye PCR Master Mix (*Helini* biomolecules), 2.5 µl of each primer pairs, and 10 µl of extracted DNA. The reactions were carried out in 25 µl volume. The amplification conditions were as follows: the first initial denaturation at 95°C for 5 min followed by 35 cycles (denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension for 72°C for 30 s, and final extension at 72°C for 5 min). All PCR reactions were carried out using Eppendorf Master Cycler. 10 µl of sterile water as a negative control and 10 µl of positive template as positive control was used (Helini biomolecules). Electrophoresis of PCR products wase performed on 2% agarose gel containing ethidium bromide and visualized under UV transilluminator.

Restriction fragment length polymorphism analysis

Amplification products of 20 *Giardia*-positive samples were performed. Genotyping by restriction fragment length polymorphism (RFLP) digestion method to distinguish between *Giardia* assemblages was achieved using *Bspl I* (10 U/µl, Thermo Fisher Scientific, USA) restriction enzyme to differentiate between Group I and II of assemblages A and B and by *Rsal* restriction enzymes (10 U/µl, Thermo Fisher Scientific) to distinguish between subassemblages BIII and BIV. Restriction condition was performed according to the manufacturer's instruction. Restriction fragment was separated by 15% polyacrylamide gel electrophoresis using 100 bp DNA ladder (*Helini's biomolecules*, Chennai, India) as size standard and visualized.[10]

Statistical analysis

The statistical analysis was performed using GraphPad Software Inc., San Diego, USA. The association between the two categorical variables was analyzed by Chi-squared test or Fisher's exact test as appropriate. Different variables were summarized using frequency tables. $P \leq 0.05$ is considered statistically significant.

Results

Out of the 400 children, *Giardia* cysts were detected in 5% of the participants (20/400) by microscopy in which 65% (13/20) were successfully assayed by PCR-RFLP-targeting *gdh* genes. The PCR product of *gdh* gene was digested by *BsPL1* and *Rsal* to differentiate between the isolates of A and B subassemblages. Out of 65% *Giardia* positive, 53.7% of BIV, 38.5% of AII and B mixed, and 7.8% of BIII assemblages were isolated [Table 1]. AI assemblage was not observed in this study. The predicted restriction fragment sizes are listed in Table 2. Higher prevalence of giardiasis was from the age group of 6–10 years and the infection rate was similar in male and female participants [Table 3]. BIV genotype was observed in the age group of 1–13 years, BIII genotype was observed in one 4-year-old patient, and mixed assemblage of BIV and B genotype was observed in 9–12-year-old children. Diarrhea and abdominal pain was the common complaint associated with giardiasis in this study.

Discussion

G. intestinalis causes human giardiasis and is the most common intestinal parasite of human especially in children.[9] It is more

Table 1: Genotypes of *Giardia lamblia* by polymerase chain reaction-restriction fragment length polymorphism targeting glutamate dehydrogenase genes

Isolate	Gdh RFLP	Sex	Age	Symptoms at present
GAP 4	AII+B	Male	11	Abdominal pain
GAP12	BIV	Male	13	Abdominal pain
GDO 74	BIV	Female	6	Diarrhea and stool odor
GDB 81	BIII	Female	4	Diarrhea and bloating
GDAP 153	AII+B	Female	9	Diarrhea and abdominal pain
GAPB 191	BIV	Female	7	Abdominal pain and bloating
GDO 227	BIV	Female	6	Diarrhea and stool odor
GD 291	AII+B	Female	12	Diarrhea
GDAP 361	AII+B	Female	10	Diarrhea and abdominal pain
GD 412	BIV	Female	9	Diarrhea
GD 478	BIV	Male	8	Diarrhea
GD 441	BIV	Male	9	Diarrhea
GDAB 112	AII+B	Male	9	Diarrhea and abdominal pain

RFLP: Restriction fragment length polymorphism, Gdh: Glutamate dehydrogenase

Table 2: *Giardia intestinalis* predicted fragment size of sub assemblages after digesting with restriction enzymes

Enzyme	Assemblages	Subassemblages	Fragment sizes (bp)
BspL1	A	AI	39, 87, 123, 145
		AII	39, 68, 77, 87, 123
	B	-	123, 287
		Mixed (AII+B)	39, 68, 77, 87, 123, 287
		Mixed (AI+B)	39, 87, 123, 145, 287
Rsal	B	BIII	130, 297
		BIV	427
		BIII/BIV	130, 297, 427

Table 3: Gender distribution of *Giardia*-positive children by microscopy

Gender	Total children	Positive	*P*
Male	189	7	0.2839
Female	211	3	

common in young children which is due to the consumption of food/water contaminated with *Giardia* cysts.

In our study, assemblage B (61.5%) was the predominant genotype observed followed by a mixed assemblage of BIV and BIII (53.7% and 7.8%). Similar observations were previously reported in which assemblage B (61%) was observed to be predominant with 38% BIII assemblages.[11,12] Although one study has reported a 93.02% prevalence of assemblages B,[13] all these studies were targeting *gdh* genes. However, several other studies have reported the assemblage A as the predominant genotype.[10,14-16] Such differences in the genotypes observed may reflect the geographical variation in the prevalence in the intestinal parasites and are hence valuable in designing effective therapeutic approaches.

The molecular genotyping in our study reveals that the human isolate of *Giardia* is associated with the assemblage A and B only which are related to human infections.[17] However, false-negative result was also reported when the *gdh* gene was amplified by nested PCR.[18] Some studies also reported that *tpi* PCR was better able to resolve mix infections with assemblage A and B since both the *gdh* and *β-giardia* PCR-RFLP typed these samples as either assemblage A or B infection alone.[12] There was also a mixed infection found in this study between AII and B. This could be due to amplification of single genotype over other at a specific locus.[19] The data from this study are in agreement with several previous studies from other geographical locations.[1,19-25] In this study, we were unable to amplify 7/20 of the *Giardia* positive by microscopy. This could be due to low DNA yield from fecal samples, either due to degradation of fecal sample and/or presence of some PCR inhibitors (lipids, hemoglobin, bile salts, polysaccharides from mucus, bacteria, and food degradation product).[26,27]

In this study, the highest prevalence of giardiasis was observed in the age group of 6–10 years. Other studies have been reported that the higher prevalence seen in 2–8 years, 1–13 years, or 0–4 years.[15,28,29]

Conclusions

The predominant *G. intestinalis* genotype found in the Sikkim, Indian region, was BIV assemblages and not anthroponotic assemblages. However, the reasons for the exact transmission of mixed assemblages are unclear. Nevertheless, studies based on different assemblage-specific genotyping based on *β-giardin, tpi*, and small subunit ribosomal RNA genes will be necessary in future.

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Conflicts of interest

There are no conflicts of interest.

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