REAL-TIME PCR/RFLP ASSAY TO DETECT GIARDIA INTESTINALIS GENOTYPES IN HUMAN ISOLATES WITH DIARRHEA IN EGYPT

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ABSTRACT: Two major genotypic assemblages of Giardia intestinalis infect humans; the nested real-time polymerase chain reaction (PCR) was used for targeting the triose phosphate isomerase (tpi) gene to detect and genotype G. intestinalis in human feces in Egypt. Among 97 fecal samples, 30 (31%) were diagnosed as giardiasis by saline wet mount microscopy after staining with Lugol’s iodine. The tpi gene was amplified from 41 (42.5%) fecal samples, of which 11 were microscopy-negative specimens. Of the total samples, 24 (58.5%) contained assemblage A group I, and 7 (17.1%) were assemblage A group II from the group of patients complaining of intermittent diarrhea. Eight (19.5%) samples contained assemblage B from patients with persistent diarrhea. Two (5%) samples had a mixture of assemblage A group II and assemblage B. The technique was able to detect as few as 20 trophozoites per PCR on fecal DNA-isolated, microscopy-negative, and quantitative (q)PCR-positive specimens; there was a higher average cycle threshold value than microscopy-positive and qPCR-positive specimens, suggesting that they represented true, low-burden infections. In conclusion, we could genotype G. intestinalis from fresh stool samples in Egypt; in infections commonly presented with intermittent diarrhea, the most prevalent genotype was assemblage A group I. The most vulnerable age group included 10- to 20-yr-old individuals.

In developing countries of Asia, Africa, and Latin America, approximately 200 million people have symptomatic giardiasis (Thompson et al., 2000; Yason and Rivera, 2007). *Giardia intestinalis* is the most prevalent cause of parasitic diarrhea in the developed world, and this infection is also very common in developing countries. Moreover, there is an association of *Giardia* species and strain assemblages with diarrhea/dysentery (Haque, 2007).

In Egypt, Foronda et al. (2008) found that assemblage B was the most prevalent (80%) genotype, with another 15% of the positive samples belonging to assemblage E, and 5% to assemblage A. Transmission of the *G. intestinalis* cyst to humans occurs mainly after ingestion of contaminated water, autoinfection, and person-to-person contact. Clinical manifestations of symptomatic giardiasis include greasy stools, flatulence, diarrhea, abdominal cramps, epigastric tenderness, as well as steatorrhea accompanied by full-blown malabsorption syndrome (Gardner and Hill, 2001). However, the majority of cases are asymptomatic or minimally symptomatic in immunocompetent individuals (Furness et al., 2000).

Species of *Giardia* can be distinguished on the basis of morphology, ultrastructural features, or the 16S rRNA sequence; there are at least 6 species, i.e., *G. intestinalis* (= *lambila*), *G. agilis*, *G. muris*, *G. ardeae*, *G. psittaci*, and *G. microti* (Monis et al., 1999). Among the 6 species, *G. intestinalis* infects humans and numerous other mammals (Thompson et al., 2000). Isolates of *G. intestinalis* are classified into 7 assemblages, based on the characterization of the glutamate dehydrogenase, small-subunit rRNA, and triosephosphate isomerase (tpi) genes (Hopkins et al., 1997; Monis et al., 1999; Sulaiman et al., 2003; Read et al., 2004). Assemblage A isolates have been further placed into subgroups I and II. Assemblage B isolates have been separated into subgroups III and IV (Thompson et al., 1994; Monis et al., 1999). Genetic assemblages C, D, E, F, and G seem to be restricted to domestic animals, livestock, and wild animals (Adam, 2001).

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were present. The isolates were genotyped based on the TPI nucleotide sequence and correlation between G. intestinalis assemblages and diarrhea.

MATERIALS AND METHODS

Human fecal specimens

Ninety-seven stool specimens were obtained from patients examined at Internal Medicine clinics in Misr University's hospitals between September 2007 and February 2008. Informed consent was obtained from all participants, and human experimentation guidelines were followed in the conduct of this research. Patients were Egyptian, aged between 4 to 65 yr; 53.6% were males and 46.4% females. All participants were examined for G. intestinalis cysts and trophozoites.

Thirty-four (35%) patients had persistent diarrhea and abdominal pain; 63 (65%) complained of intermittent diarrhea, flatulence, epigastric tenderness, crampy (painful involuntary contractions) abdominal pains, and copious light-colored greasy stools. Stool samples were collected fresh and divided into 2 parts; the first part was examined directly by the saline wet mount microscopy technique after staining with Lugol’s iodine to detect G. intestinalis infection. Trophozoites were counted in a hemocytometer, sedimented, washed in sterile phosphate-buffered saline (PBS), and spiked into 200-ng aliquots of parasite-free stool. The second part was stored without preservatives at −20 C for molecular diagnosis.

DNA extraction

DNA was extracted using the commercial QIAamp method (QIAGEN, Valencia, California). Fecal specimens were washed twice with sterile PBS and centrifuged for 5 min at 18,000 g; the fecal pellet was suspended in 0.6 ml of ATL lysis buffer (provided with the QIAamp stool kit, QIAGEN) and 40 ml of proteinase K and was incubated in a 55 C water bath for 6 hr. The sample was subjected to 3 freeze-thaw cycles and then incubated at 55 C overnight. The DNA was extracted following the manufacturer’s procedure for the QIAamp DNA stool kit (QIAGEN). DNA was eluted from the silica gel column using 2 rounds of 100 ml of nuclease-free water. The concentration of extracted DNA was measured by a spectrophotometer at 260 nm, and then the sample was stored at −20 C until use.

PCR amplification

The procedure followed was as described by Amar et al. (2003). The reaction mixtures contained 10× PCR buffer (Pharmacia Biotech, Freiburg, Germany), 10 mM (each) deoxynucleoside triphosphates (dNTPs), and 2.5 U of Taq polymerase (HotStarTaq, QIAGEN). Two sets of 4 primers were used to amplify G. intestinalis assemblages A and B. Five microliters of fecally extracted DNA was used, and amplification was performed on PCR (real-time hot-start PCRs using a LightCycler (Biomera, Göttingen, Germany)). Amplification was performed in 2 phases. A duplex phase I PCR was performed using primers designed to amplify fragments of the tpi gene of G. intestinalis of 576 base pairs (bp) from assemblage A (primers TPIA4F/TPIA4R) and 210 bp from assemblage B (primers TP1B4F/TP1B4R). The duplex reaction was performed in a 10-μl volume with 5 μl of DNA in 1× PCR buffer, 2 mM MgCl₂, 0.25 mM (each) dNTPs, 0.3 μM (each) primer, and 0.5 U of Taq DNA polymerase (all reagents from Pharmacia Biotech). Samples were subjected to an initial denaturation of 94 C for 1 min, 25 cycles of 94 C for 20 sec, 50 C for 30 sec, 72 C for 1 min, and a final extension at 72 C for 5 min. Each test batch contained a maximum of 20 samples plus 1 positive control and 1 negative control (water).

Sensitivity of nested real-time PCR (TPILC-PCR)

Phase I of the TPILC-PCR was performed in a conventional thermocycler (Biomera), and only the nested phase was adapted to the LightCycler system. Two separate phase II PCRs, with inner forward (IF) and reverse (IR) primers, were devised to amplify the fragments of the G. intestinalis tpi gene of 452 bp from assemblage A (primers TPIA4IF/ TPIA4IR) and 141 bp from assemblage B (primers TP1B4IF/TP1B4IR). Both phase II reactions were performed as real-time hot-start PCRs using a LightCycler. The reaction consisted of the phase I duplex PCR product, 2 mM MgCl₂, 1 μM (each) primer (IF/IR), and Master Mix (Fast Start DNA Master SYBR Green I kit; Roche Molecular Biochemicals, Mannheim, Germany) in a volume of 50 μl. Cycling conditions were 95 C for 8 min followed by 40 cycles of 95 C for 15 sec, 58 C for 3 sec, and 72 C for 10 sec, with a transition rate of 20 C sec⁻¹. Fluorescence readings were taken after each extension step.

Gel electrophoresis and RFLP

Real restriction sites were identified from an alignment of the tpi gene of G. intestinalis assembly A to distinguish between sub-genotypes groups I and II. The predicted restriction fragments were 437 and 15 bp for group I and 235, 202, and 15 bp for group II. RFLP analysis was performed by digesting 5 μl of phase II product with 5 U of restriction enzyme in 1× enzyme buffer (Invitrogen, Carlsbad, California) in a final volume of 30 μl for at least 4 hr at 37 C. Restriction fragments were separated in 2% agarose/ethidium bromide gels by horizontal electrophoresis and examined by UV transillumination (Fig. 1).

DNA sequencing

PCR products were purified using a StrataPrep PCR purification kit (Stratagene, La Jolla, California). Sequencing of PCR products (sense and antisense) was performed at the Molecular Biology Unit, Medical College, Cairo, Egypt, using an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, California) and appropriate IF and IR primers.

Statistical analysis

Data were analyzed using Statistical Package for Social Science, version 8 (SPSS Inc., Chicago, Illinois), and Student’s t-test. A P value <0.05 was considered significant.

RESULTS

Of the 97 specimens examined by microscopy, 30 were positive and 67 were negative for G. intestinalis. Parasite loads in these samples ranged from very heavy to very light. Nested real-time PCR showed 30 (58%) males and 11 (24.4%) females infected with G. intestinalis. From the present study, the highest percentage of infection was found in the 10- to 20-yr-old age groups (56.3%) (Table I). Overall, 41 (42.3%) of patients reported diarrhea as the
main gastrointestinal manifestation (32 patients had intermittent type and 9 patients had persistent type). Abdominal pain was observed in 95% of patients. Other manifestations, such as flatulence (11/41; 26.8%), weight loss (4/41; 9.8%), anorexia and nausea (9/41; 22%), and fatigue (4/41; 9.8%), were reported; approximately 25% of the patients indicated more than 1 complaint. A correlation was observed between assemblages of \textit{G. intestinalis} detected and the presence of diarrhea. Patients who had intermittent diarrhea (24/41; 58.5%) had assemblage A group I, whereas 7/41 (17%) patients had assemblage A group II, and 1 patient had mixed assemblages A and B. This is a significant difference ($P < 0.05$) versus patients with persistent diarrhea, which was reported in 8/41 (19.5%) patients who had assemblage B, along with 1 patient who had both assemblages A and B.

The RFLP fragments of the TPIA-LC-PCR product from 24 fecal samples were identical to the \textit{G. intestinalis} assemblage A group I sequence (L02120). Analysis of the products amplified from 7 fecal sample patterns were identical (referring to positive PCR/RFLP fragments analysis) to the \textit{G. intestinalis} assemblage A group II sequence (U57897). TPIBLC-PCR products from 8 fecal samples showed patterns identical to the \textit{G. intestinalis} assemblage B sequence (L02116 and AF069561). The banding pattern confirmed that 2 DNA extracts contained both assemblages A group II and B and also confirmed the specificity of the PCR assays.

For confirmation, 3 of the PCR products (2 from assemblage A and 1 from assemblage B) were sequenced and revealed complete identity to the published sequence (GenBank L02120 and U57897, respectively) and assemblage B (L02116 and AF069561). The TPILC-PCR assay demonstrated the \textit{tpi} gene in 42.3% of the samples (41 of 97), of which 30 were microscopy positive, and 11 were microscopy negative. There were delayed cycle threshold (CT) values versus the microscopy-positive and qPCR-positive specimens, suggesting that the microscopy-negative and qPCR-positive specimens represented true low-burden infections.

The sensitivity of the assay using the QIAamp extraction method to detect \textit{G. intestinalis} DNA in stool was \( \leq 20 \) trophozoites per reaction for assemblage A (\( CT = 37.4 \)) and \( \leq 200 \) trophozoites per reaction for assemblage B (\( CT = 35.0 \)). These results suggest that the DNA yield was limited as opposed to PCR inhibition. Regarding the specificity of nested real-time PCR, our assay was first evaluated by primers to a BLAST test (http://www.ncbi.nlm.nih.gov/BLAST/). BLAST tests returned 100% sequence matches with the A-rev primer. The specificity was then examined by performing PCR assays. The specificity of the assemblage A and B was tested on genomic DNA from \textit{G. intestinalis} assemblages isolated from stool samples with up to 200 assemblage A or B trophozoites per reaction. The predicted RFLP product (437; 15 bp) was obtained from assemblage A subtype I and assemblage A subtype II (235, 202; 15 bp) and 141 bp for assemblage B. No amplification was detected by qPCR or gel electrophoresis on discordant stool samples.

**DISCUSSION**

In Egypt, epidemiological studies of patients with diarrhea (Zaki et al., 1986) found \textit{G. intestinalis} in 44% of the population studied in rural areas, whereas Shukry et al. (1986) reported a prevalence of 33% in Cairo residents. Curtale et al. (1998) and Fawzi et al. (2004) detected \textit{G. intestinalis} in 24.7% of fecal samples in Behera Governorate and in 10.4% in El-Prince (Alexandria), both in Egypt. Antibodies against \textit{G. intestinalis} were reported by Azab et al. (1991) from Egyptian women, whereas Mahmoud et al. (2001) found seropositivity to \textit{G. intestinalis} in infants in Bilbeis.

In the present study, the distribution of major assemblages in sporadic human giardiasis in Egypt. Our observations revealed that the majority of giardiasis isolates were assemblage A genotype (31/41; 76%), corresponding to the findings of an Italian study that reported 80% assemblage A (Cacciò et al., 2002). In addition, intermittent diarrhea is the common presentation in 78% of positive \textit{G. intestinalis} patients (31 patients had assemblage A, and 1 patient had mixed assemblage A and B), a significant result ($P < 0.001$) compared with other manifestations. Recently, it was reported that assemblage B was seen in patients with persistent diarrhea, whereas assemblage A was seen mostly in patients with intermittent diarrhea (Homan et al., 2003); our results reflect this pattern. The present study also revealed that abdominal pain was a significant correlate ($P < 0.0001$) compared with other presenting symptoms. However, 25% of our patients had more than one complaint, but this was not significant ($P > 0.05$).

In the present study, the significance of a predominant subgenotype (assemblage A) is unclear. Differences in the prevalence of assemblages A and B may be attributed to the geographic locations of the patients studied. It is tempting to conclude a common source of human infection was responsible for the wide occurrence of subgenotype assemblage A group I, i.e., contamination of public water with raw sewage from animal and human sources. It is also possible that subgenotype group I of assemblage A is more infectious to humans than other \textit{Giardia} parasites.

The sensitivity of the fully nested reaction was 0.5–5 copies of \textit{tpi} in our work. Moreover, the assays used were quick, requiring only 1 hr 50 min for the LightCycler assay to be completed. In addition, nested real-time PCR is specific and discriminated between \textit{G. intestinalis} assemblages A and B. The assay consisted of an initial multiplexed block-based amplification, followed by 2 separate real-time PCR assays specific for assemblages A and B using a LightCycler and SYBR Green I to identify PCR products by melting point analysis.
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