Original Article

Genotyping *Giardia intestinalis* by Using DNA Extracted from Long-Term Preserved Human Specimens Stained with Chlorazol Black E

Yoshie Nishida1,3, Norihito Morimoto*, Masataka Korenaga2, Yutaka Komatsu1, Hiroaki Takeuchi1, Yoshihisa Matsumura1, and Tetsuro Sugiuira1,3

1Department of Clinical Laboratory, Kochi Medical School Hospital; 2Parasitology, and 3Clinical Laboratory Medicine, Kochi Medical School, Kochi University, Kochi 783-8505, Japan

SUMMARY: *Giardia intestinalis* is a parasitic protozoan that causes diarrhea and abdominal pain in humans. Studies of the *Giardia* genotypes are thought to be important for understanding their infection routes and prevalence. However, few have reported pathogen genotyping in human giardiasis cases in Japan. In this study, we genotyped *G. intestinalis* by using DNA extracted from chlorazol black E-stained fecal samples from patients. The triosephosphate isomerase gene was amplified from 21 (91.3%) of 23 human fecal samples. Twelve (52.2%) of pathogens detected were of the genotype A, and 9 (39.1%) of the genotype B. A restriction fragment length polymorphism analysis showed that all genotype A found in the present study were of the genotype AI, which were presumed to be zoonotic. The source of *Giardia* infections was unclear in the present study. However, patients' histories of international travel appeared not to be associated with the *Giardia* genotypes. Thus, most cases were thought to be acquired sporadically and domestically.

INTRODUCTION

*Giardia intestinalis* (syn. *G. lamblia* and *G. duodenalis*) is a protozoan that parasitizes the small intestines and bile ducts of mammals (1). *G. intestinalis* infection is acquired from water or cysts-containing food, and it can spread from person to person. Prevalence rates for giardiasis are 2–7% in developed countries and 20–30% in most developing countries (2). Waterborne outbreaks have been reported in several industrialized countries (3–5), but yet to be reported in Japan. In Japan, approximately 100 cases of giardiasis are reported annually (6). However, sources of infection were unclear in many cases.

Traditionally, identification of *G. intestinalis* is based on its morphological characteristics in fresh fecal samples using conventional methods, such as direct smears, formalin-ethyl acetate concentrations, and cyst smears stained with chlorozol black E (CBE) or hematoxylin. Recently, molecular classification has been used to investigate pathogenesis and host range of *G. intestinalis* (2,7). Host specificities of several *G. intestinalis* genotypes have been assessed using PCR and restriction fragment length polymorphism (RFLP) analyses. *G. intestinalis* is classified into at least 7 genotypes, A–G (8–10). All *G. intestinalis* isolates from humans belong to the genotype A (including genetic groups AI and AII) and genotype B. Infections of genotypes AI and B occur in both humans and animals. However, genotype AII is generally found only in humans (11).

We have previously demonstrated that DNA of the parasite remains stable in CBE-stained smears (12). Because specimens were fixed in methanol, nuclear extracts from specimens stored for extended periods could be amplified by PCR. In this study, we analyzed the genotypes of *G. intestinalis* DNA amplified from long-term preserved specimens stained with CBE. We discuss potential infection routes of the parasite based on the genotyping results.

MATERIALS AND METHODS

Samples: Twenty-three *G. intestinalis*-positive human fecal specimens stained with CBE were collected at Kochi Medical School Hospital, Kochi Prefecture, Japan from 1996 to 2009. Cysts and/or trophozoites were confirmed microscopically. The specimens were stored at room temperature until use.

DNA extraction from CBE-stained smears: To avoid contamination, slide surfaces were washed with a DNA removal reagent (DNA Away, Molecular Bioproducts, San Diego, CA, USA). Stained smears were then placed into 50-mL conical tubes containing xylene, and glass coverslips were removed. Smears were subsequently hydrated through a graded series of ethanol (100%, 95%, 90%, 80%, 70%, 60%, and 50%), and washed 3 times in TE buffer. Specimens were collected from slides with a scalpel, transferred into 2-mL tubes, and centrifuged at 13,000 × g for 5 min. The resulting cell pellets were then subjected to 5 freeze-thaw cycles at −80°C in a deep freezer and 95°C in a water bath, respectively. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer’s instructions.

PCR and RFLP analyses: Sets of PCR primers used in the present study are shown in Table 1. A semi-nested PCR targeting the triosephosphate isomerase (*tpi*) gene...
was performed using primers designed by Amar et al. (13), with some modifications. Briefly, the first PCR mixture contained 6 μL distilled water, 2 × PCR buffer for KOD FX (Toyobo Life Science, Osaka, Japan), 0.4 mM dNTPs, 1 U KOD FX DNA polymerase (1 μL; KFX-101, Toyobo Life Science), 5 μL template, and 15 pmol of each primer (for genotype A: mTPIA-FI and mTPIA-R, generating a 576-bp product; and for genotype B: mTPIB-FI and mTPIB-R, generating a 208-bp product), in a total volume of 50 μL. All reactions had an initial denaturation step at 94°C for 2 min, followed by 30–40 cycles of 98°C for 10 s, 63°C for 30 s, and 68°C for 15–45 s. The second PCR mixtures for genotypes A or B contained the PCR mix described above, 15 pmol of each primer (genotype A: mTPIA-FII and mTPIA-R, generating a 140-bp product; and for genotype B: mTPIB-FII and mTPIB-R, generating a 476-bp product), in a total volume of 50 μL. All reactions were performed with the thermal cycling protocol described above. Both positive and negative controls were included in each PCR to validate the results. Amplifications were assessed by electrophoresis on agarose gel containing ethidium bromide. An RFLP analysis was performed by digesting 10 μL of the TPIA-PCR (genotype A) products with 5 U of Rsal (New England BioLabs, Ipswich, MA, USA), following the manufacturer’s instructions.

Nucleotide sequences were confirmed with a sequence analyzer. Briefly, bands were excised from agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified fragments were sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were processed using the Sequence Scanner Software v1.0 (Applied Biosystems) and aligned against the following GenBank entries: L02120 (G. intestinalis genotype A1), U57897 (G. intestinalis genotype AI), and L02116 (G. intestinalis genotype B). Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (14) and aligned with the ClustalX software (15).

**RESULTS**

The tpi gene was amplified from 21 of 23 known *G. intestinalis*-positive CBE specimens (91.3%). Overall, 9 of the 23 samples (39.1%) were determined as genotype B and 12 (52.2%) were determined as genotype A. An RFLP analysis showed that all genotype A pathogens were of the AI genotype. Representative results are shown in Fig. 1.

We performed a BLAST search of the *G. intestinalis* genome using the identified AI and B genotype as the query. The identified sequences were verified as L02120 and L02116, encoding the tpi gene of genotype AI and B, respectively.

We investigated histories of the 23 patients in international travel and animal keeping. Three of the 9 patients infected with *G. intestinalis* genotype B had travelled abroad to Hong Kong 2 years before their admission to hospital, to Canada 7 years before admission, or to Korea and Taiwan 20 years before admission, whereas 1 of 12 patients with genotype A had travelled to Europe 3 years before admission. However, most patients had not travelled abroad. Nine of the 23 patients kept animals at home (Table 2).

**DISCUSSION**

Although giardiasis has been thought to be a predominantly imported infectious disease in Japan, our present results suggest that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically. Medical history interviews conducted in this study indicated that most infections appear to be acquired domestically.

Genotypes of the parasites identified in the present study were mainly of the zoonotic types, genotypes AI and B, while no anthroponotic genotype AI was detected. Among patients tested, 1 was involved in cattle production and nearly half of the patients kept companion animals at home (Table 2). It has been suggested that transmission of *Giardia* from companion animals is relatively important in countries with high hygiene standards, such as Japan (16). It has been shown that the prevalence of *Giardia* is 14.5% in household dogs and 40% in household cats in Japan, as assessed by microscopic examinations and an enzyme-linked immunosorbent assay technique, respectively (17,18). Despite a relatively low prevalence of *Giardia*-positive dogs, our
Fig. 1. Results of the PCR and RFLP analysis. (A) Electrophoresis of the PCR products on agarose gel containing ethidium bromide. Heminested PCR targeting the triosephosphate isomerase (tpi) gene. (A: 476 bp; B: 140 bp). M: marker; lanes 1–13: specimens; NC: negative control; PC: positive control. (B) RFLP analysis after digestion of 10 µL of the TPIA-PCR product with 5 U of RsaI for 4 h at 37°C. RsaI restricts the GTAC site. Digestion of genotype A1 (476 bp) with RsaI generated products of 437 bp. M: marker; lanes 3–11: TPIA-PCR-positive samples digested with RsaI; PC: positive control (not digested).

study supported the notion that these dogs may pose as significant risk factors for giardiasis as they can transmit *Giardia* cysts to humans (19). Sotiriadou et al. (20) reported that genotype A of *G. intestinalis*, a zoonotic species, was detected more frequently than genotypes C and D in infected dogs in southern Germany. They suggested that household animals are potential reservoirs of *Giardia* for human infection. Several other studies have also implicated companion animals as potential sources of *Giardia* infection (21–25). In contrast, anthropopotic transmission is considered to be a major route of infection in developing countries such as Malaysia and Yemen (26,27).

Our previous study showed that the prevalence rate of *G. intestinalis* in hospitalized patients with mild or no diarrhea in southwestern Japan was 0.6% (28). However, the exact prevalence, as well as the relationship between *G. intestinalis* genotypes in humans and animals in Japan is still unclear. This is partly due to the relative difficulty of obtaining *G. intestinalis* isolates from humans by the ordinary methods.

In conclusion, our study suggests that most patients in Japan acquire *G. intestinalis* infection domestically and that zoonotic genotypes are involved in these domestic infections. Further studies are required to elucidate the genotypes of human and animal isolates of *G. intestinalis* to further address the host/parasite relationship.

Conflict of interest None to declare.

REFERENCES


