Experimental Parasitology 134 (2013) 141–147

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Molecular characterization of *Giardia duodenalis* in Yemen

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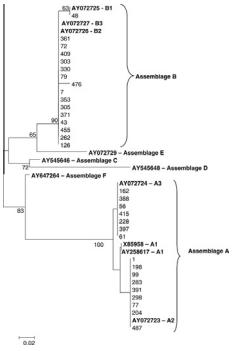
HIGHLIGHTS

- *Giardia duodenalis* genotype and subtype were determined among Yemeni outpatients.
- Assemblage A was identified in 66% of positive samples.
- Assemblage B was identified in 34% of positive samples.
- Further subtyping analysis identified subtypes A2 and A3.
- Data suggest the potential role of anthroponotic transmission.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 September 2012
Received in revised form 20 November 2012
Accepted 2 March 2013
Available online 21 March 2013

Keywords:
Giardia duodenalis
Genotyping
Yemen

ABSTRACT

Giardia duodenalis is an important intestinal protozoan in Yemen with infection rates ranging from 18% to 27%. To date, there has been no genotyping study to provide a better understanding of the transmission dynamic. This study was conducted to genotype and subtype *G. duodenalis* in Yemen. Stool samples were collected from 503 Yemeni outpatients between 1 and 80 years old, including 219 males and 284 females. *Giardia* cysts were detected via microscopy after the formal-ether concentration. Genotyping of *Giardia* was carried out using PCR and sequence analysis of the 16s rRNA and *b*-giardin genes. Of the 89 microscopy-positive *Giardia* samples, 65 were successfully sequenced, of which 66% (43 of 65) were identified as *G. duodenalis* assemblage A and 34% (22 of 65) as assemblage B. Further subtyping analysis based on *b*-giardin gene identified the presence of subtypes A2 and A3, which belong to the anthroponotic subassemblage AII. Data of the study suggest that anthroponotic transmission played a potential role in the transmission of giardiasis in the community. However, further genotyping and subtyping studies of specimens from humans and animals living in the same households are needed for a more definitive understanding of giardiasis transmission in Yemen.

1. Introduction

Giardia duodenalis is a flagellated protozoan infecting humans and a wide range of animals worldwide, especially in developing countries (Islam and Meyer, 1990). Although an estimated 200 million individuals acquire symptomatic giardiasis every year, the majority of cases are asymptomatic (WHO, 1996). Chronic giardiasis may cause malnutrition, growth retardation and impairment in cognitive functions in early childhood (Ortega and Adam, 1997).

Genotyping studies showed that *G. duodenalis* is a species complex comprising of eight assemblages A–H (Feng and Xiao, 2011). Among them, assemblage A infects humans and a broad range of animals, including farm animals and companion animals, thus this assemblage has been considered having the most zoonotic potential (Feng and Xiao, 2011). In endemic areas, where humans and

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http://dx.doi.org/10.1016/j.exppara.2013.03.001

animals live in close contact, transmission from humans to animals or vice versa has been suggested (Traub et al., 2004). One such group of animals are lambs, which have been found to be frequently infected by zoonotic potential assemblages (Lebbad et al., 2010). Assemblage B infects humans (Caccio et al., 2005) and has been reported less frequently in animals (Thompson, 2004). Assemblages C–H are largely host specific (Feng and Xiao, 2011).

Subtyping of *G. duodenalis* based on *b*-giardin and other genes have identified most of the six assemblage A subtypes (i.e., A1–A6) which belong to the subassemblages AI, AII, and AIII (Feng and Xiao, 2011; Caccio et al., 2008). Of these, A1 is considered most likely to cause zoonotic transmission (Feng and Xiao, 2011; Caccio et al., 2008). Within assemblage B, subtypes B1, B3, B4, B5 and B6 were identified in one study, with only assemblage B3 being detected in both humans and animals (Lalle et al., 2005). Several studies have tried to correlate between *G. duodenalis* assemblages and clinical symptoms but the issue remains controversial (Mahdy et al., 2009).

In Yemen, *Giardia* has been identified as one of the most common intestinal parasites. A recent study in Yemen, which was conducted in 2009 reported a 17.7% prevalence of giardiasis and implicated drinking untreated water as a risk factor for infection (Alyousefi et al., 2011). However, there has been no genotyping study of *G. duodenalis* in Yemen. This study was aimed to genotype and subtype *G. duodenalis* in Yemenis seeking medical services.

2. Materials and methods

2.1. Sample sources and ethic clearance

Samples were collected from two hospitals and one clinical center in Sana'a, the capital city of Yemen, including the Al-Jomhury Hospital, Al-Kuwait Hospital and Alzahrawy Clinical Center. Patients who were referred to the parasitology laboratory during the period from December 2008 to March 2009 were asked to participate in the study after a clear explanation of the study objectives and written consent. If the patients were children, informed consents were taken from their parents. The study protocol was approved by the research and ethics committee of University of Malaya Medical Centre (MEC RF. No: 782.9). Permission was also obtained from the hospital authorities before the commencement of the study.

2.2. Questionnaire and microscopy

Collection of samples was done simultaneously with data gathering via a pre-tested questionnaire, which included sociodemographic information such as age, gender and residence, health practices (i.e., personal awareness of food and water handling besides the adoption of hygiene), behavioral habits (i.e., washing hands before eating, washing hands after defecation, eating with hands, and washing fruits and vegetables), and health conditions including the occurrence and history of symptoms (e.g., diarrhea, nausea, vomiting, and abdominal pain). Diarrhea was defined as unusually loose stools of $\geqslant 3$ times/day for both children and adults (WHO, 1988). Environmental factors such as water supply, provision of sanitation system, and direct contact with animal were also included. Age of participants was categorized into two groups: $\leqslant 12$ years and >12 years as in a previous study (Mahdy et al., 2008). For the identification of *Giardia* cysts, two to five grams of stool were mixed with distilled water, sieved through double layers of wetted gauze and centrifuged at 2000 rpm for 5 min. Ten milliliters of 10% formalin and 3 milliliters of ether were added to the pellet, mixed vigorously and centrifuged at 2000 rpm for 5 min (Suwansaksri et al., 2002). A wet mount was prepared from the sediment of stool samples and iodine staining. The wet mount preparation was examined under a light microscope at 100× and 400× magnifications.

2.3. Genotyping and subtyping of G. duodenalis

Prior to DNA extraction, the fecal specimen was emulsified with distilled water, sieved through two layers of cotton gauze to remove the debris and centrifuged at 2000 rpm for 5 min. Genomic DNA was extracted from the pellet using PowerSoil extraction mini kit according to the manufacturer's instructions (PowerSoil Isolation Kit, MO BIO Laboratories, cat. No. 12888-10) and kept at −20 °C until further use. A fragment (200 bp) of the SSU rRNA was amplified by nested PCR using primers RH11 and RH4 in the primary PCR (Hopkins et al., 1997) and primers *Giar*-F and *Giar*-R in the secondary PCR (Read et al., 2002). Primary and secondary PCR were performed in a total of 25 μl reaction mixture containing 12.5 pmol of each primer (Research Biolab, Singapore), 2U Taq polymerase (New England Biolabs, cat. No. M0267L, Ipswich, USA), 2× PCR ThermoPol buffer (New England Biolabs, cat. No. M0267L, Ipswich, USA), 200 μM dNTPs (Fermentas, cat. No. #R0192, Ontario, Canada), 2 mM $MgCl_2$ (Fermentas, cat. No. #R0971, Ontario, Canada). 5% Dimethyl sulfoxide (DMSO) (Sigma, cat. No. 673439, USA), and 400 mg/ml bovine serum albumin (BSA) (New England biolabs, cat. No. #B14, Ipswich, USA). Two microlitre of DNA template was used in both primary and secondary PCRs. Samples were incubated in MyCycler thermal cycler (Bio-Rad, cat. No. 170-9705, Hercules, USA) under the following conditions: denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturing for 20 s at 96 °C, annealing for 20 s at 59 °C and extension for 20 s at 72 °C, followed by a final extension at 72 °C for 7 min.

A semi-nested PCR was used in the amplification of the partial *b*-giardin gene. In the primary PCR, a 753-bp fragment was amplified using primers G7 and G759, whereas in the secondary PCR, a 390-bp fragment was amplified using primers G376 and G759 (Caccio et al., 2002). The PCR mixture for primary and secondary PCR consisted of 1× buffer containing 1.5 mM $MgCl_2$ (Fermentas, Ontario, Canada), 200 μM of dNTP (Fermentas), 0.2 μM of each primer, 2 U of Taq polymerase (New England Biolabs, Ipswich, USA), 400 mg/ml of non-acetylated bovine serum albumin (New England Biolabs) and 2 μl of purified DNA in a final volume of 50 μl. The PCR cycling conditions for primary and secondary PCR were as follows: after an initial denaturation of 5 min at 95 °C, 40 cycles of amplification were run, each consisting of 30 s at 95 °C, 30 s at 65 °C and 60 s at 72 °C, followed by a final extension of 7 min at 72 °C. The PCR products were subjected to electrophoresis on 2% agarose gels and stained with Syber stain. Genomic DNA positive for *G. duodenalis* was used as positive control in each run of PCR. Distilled water was used as negative control.

2.4. DNA sequencing and phylogenetic analysis

Positive bands were excised from the gel and purified using QIAquick Gel Extraction Kit (QIAgen, Germany), according to the manufacturer's instructions. DNA sequencing was conducted for the two genes in both directions using the secondary PCR primers. The sequencing was carried out using a 3130 × 1 Genetic Analyzer (Applied Biosystems, Foster City, USA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were edited manually and the consensus sequences were created using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The consensus sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool