Molecular characterization of *Giardia lamblia* in children less than 5 years of age with diarrhoea attending the Bengo General Hospital, Angola

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Background: *Giardia lamblia* is a pathogenic intestinal protozoan with high prevalence in developing countries, especially among children. Molecular characterization has revealed the existence of eight assemblages, with A and B being more commonly described in human infections. Despite its importance, to our knowledge this is the first published molecular analysis of *G. lamblia* assemblages in Angola.

Methods: The present study aimed to identify the assemblages of *G. lamblia* in children with acute diarrhoea presenting at the Bengo General Hospital, Angola. A stool sample was collected and microscopy and immunochromatographic tests were used. DNA was extracted and assemblage determination was performed through amplification of the gene fragment ssu-rRNA (175 bp) and β-giardin (511 bp) through polymerase chain reaction and DNA sequencing.

Results: Of the 16 stool samples screened, 12 were successfully sequenced. Eleven isolates were assigned to assemblage B and one to assemblage A. Subassemblage determination was not possible for assemblage B, while the single isolate assigned to assemblage A was identified as belonging to subassemblage A3.

Conclusion: This study provides information about *G. lamblia* assemblages in Bengo Province, Angola and may contribute as a first step in understanding the molecular epidemiology of this protozoan in the country.

GenBank accession numbers for the ssu-rRNA gene: MF479750, MF479751, MF479752, MF479753, MF479754, MF479755, MF479756, MF479757, MF479758, MF479759, MF479760, MF479761.

GenBank accession numbers for the β-giardin gene: MF565378, MF565379, MF565380, MF565381.

Keywords: Angola, Children, Diarrhoea, Genotyping, *Giardia lamblia*, Hospital

Introduction

*Giardia lamblia* is a common intestinal parasite infecting a broad range of vertebrate species, including humans.¹ This parasite has a global distribution and it is estimated that 280 million people are infected worldwide,²,³ with 200 million people presenting symptomatic giardiasis in the developing countries.²⁴ Children living in developing countries with poor hygiene and
sanitation conditions are more vulnerable to the clinical consequences of *G. lamblia* infection. The harmful effect of giardiasis on growth and development in children has been observed in several studies and the potential effects of chronic malnutrition on cognition, intelligence and psychomotor development have also been described. Several studies have shown that malnutrition in children can be an important consequence of *Giardia* infection. However, there are few published studies in recent years exploring the association of the assemblage of *G. lamblia* with infant nutritional status.

Currently *G. lamblia* is considered a species complex, comprising eight assemblages (A–H). The majority of these assemblages are host specific, but only assemblages A and B are known to infect humans, with assemblage B being described as more common. A recent study reported for the first time assemblage E in humans in Australia. However, the number of molecular epidemiological studies of giardiasis in humans is not enough to determine geographic or socio-economic differences in the distribution of assemblages A and B.

The severity of the disease is determined by the interaction among parasite’s virulence, host’s nutritional and immunological status, nature of the intestinal microflora and the presence or absence of other pathogenic intestinal agents. Although the different assemblages of *G. lamblia* may eventually produce different toxins or metabolic products that contribute to their pathogenicity or differences in antigenic variation and host specificity, studies on the possible association between the genetic groups of *G. lamblia* and their virulence (defined by the probability of causing diarrhoea and other clinical symptoms) continue to show inconsistent results. Although several studies have shown a correlation between *G. lamblia* infections belonging to assemblage B with more severe symptoms, others have correlated the most severe symptoms with assemblage A.

An interesting observation raised by Almeida et al. was that there seems to be a relationship between the molecular marker used in the study and the assemblage believed to be more aggressive. These investigators found that almost all genotyping studies based on the ssu-rRNA and tpi genes support the idea that assemblage A is associated with symptomatic disease, whereas studies based on the bg and adh genes associated the symptomatology with assemblage B.

The occurrence and spread of giardiasis in human populations is an emerging public health problem around the world. Molecular studies are essential to clarify the importance of local *Giardia* assemblages.

In Angola, there is limited information available concerning infection with intestinal parasites and only in recent years have some studies been published. Two of these studies report *G. lamblia* as being the most prevalent intestinal protozoan in children from the province of Bié and the city of Lubango (Huila province). We have no knowledge of previous studies on molecular characterization of *G. lamblia* carried out in Angola.

The aim of the present study was to perform a genetic characterization of *G. lamblia*, using polymerase chain reaction (PCR) and DNA sequencing, in children with diarrhoea attending the Bengo General Hospital.

### Materials and methods

#### Population and study design

This study is part of a cross-sectional study conducted between September 2012 and December 2013 at the Bengo General Hospital, located in Caxito, the capital city of Bengo Province, 60 km northeast of Luanda, Angola. The study aimed to investigate the aetiology of diarrhoea in children younger than 5 years attending the paediatric emergency service or the hospital outpatient paediatric unit with diarrhoea (three or more loose or liquid stools per day). Children receiving antibiotics or antiparasitic drugs were excluded in order to avoid false negatives. A survey including sociodemographic characterization, information on breastfeeding practices, water source and sanitation conditions was applied by the clinical staff. Symptomatology in the previous 10 days (diarrhoea, vomiting, fever and bloody diarrhoea) was reported by caregivers. Anthropometric measurements (weight and length/height) were assessed to calculate anthropometric indices expressed as a Z-score for each child.

The study protocol was approved by the Ethics and Committee of the Angolan Ministry of Health and the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Portugal. Written informed consent and voluntary consent was obtained from parents or legal guardians prior to the inclusion of each child.

#### Stool sample collection and initial diagnosis of *G. lamblia*

A single stool sample per child was collected to a sterile container provided by the clinic staff. Once delivered to the laboratory, stool samples were immediately processed for microscopic identification of cysts and/or trophozoites of *G. lamblia* (direct examination with saline and iodine and a concentration method (ParasiTrap system, Biosepar, Mühldorf, Germany)) followed by the detection of Giardia antigen through immunochromatographic rapid tests (RIDAQUICK Cryptosporidium/Giardia Combi, R-Biopharm, Darmstadt, Germany). Microscopy and immunochromatographic rapid tests were performed in 344 and 338 children, respectively. Positive samples by at least one of the methods applied were preserved at −20°C for DNA extraction (Figure 1).

#### DNA extraction

DNA was extracted from *G. lamblia*-positive stool samples preserved at −20°C using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, except for the final step where 100 μl of the elution solution were used.

#### DNA amplification

The DNA of the extracted samples was amplified through PCR, using primers targeting the small subunit ribosomal RNA (ssu-rRNA) (Figure 1). The primers used were RH11: 5’-CAT CCG GTC GAT CCT GCC-5’ and RH4: 5’-AGT CGA ACC CTG ATT CTC GGC CCA GG-3’ for the first reaction and GiarF: 5’-GAC GCT CTC CCC AAG GAC-3’ and GiarR: 5’-CTG CGT CAC GCT GCT CG-3’ for the
Figure 1. Molecular characterization of *Giardia lamblia* detected by microscopy and/or rapid antigen test in stool samples from children with diarrhoea attending the Bengo General Hospital, Angola.
secondary PCR. All samples successfully amplified for ssu-rRNA were posteriorly amplified for the β-giardin (bg) loci (Figure 1). The primers used were G7: 5′-AAG CCC GAC CTC ACC CGC AGT GC-3′ and G759: 5′ G AG GCC GCC CTG GAT CTT CGA GAC GAC-3′ for the first reaction and G8: 5′-GAA CCA AGC AGA TCG AGG TTC G-3′ and G9: 5′-CTC GAC GAG CTT CGT GTT-3′.

Amplification reactions were performed using 2 μl of DNA template in a final volume of 25 μl, using illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, UK). Both positive (DNA isolated from the Portland-1 strain; ATCC 30888D, ATCC-LGC Promochem, Manassas, VA, USA) and negative controls (no template added) were included in each series of PCRs. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

DNA sequence analysis

For sequence analysis, PCR products of amplified samples were purified using illustra GFX PCR DNA and the Gel Band Purification Kit (GE HealthCare) according to the manufacturer’s instructions. DNA sequencing reactions were carried out in both directions for ssu-rRNA (175 bp) and β-giardin (511 bp) PCR-generated fragments.

Sequences obtained (75.0% [12/16] for ssu-rRNA and 80.0% [4/5] for β-giardin) were aligned with previously published sequences of G. lamblia isolates available in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using BLAST for assemblage determination and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for subassemblage determination.

Results

Microscopic identification and antigen detection of G. lamblia was performed in a total of 338 children, of whom 73 (21.6%) were positive: 40 (54.8%) were only detected through immunochromatographic tests, 2 (2.7%) only by microscopy and 31 (41.2%) positive: 40 (54.8%) were only detected through immunochromatographic tests. However, the isolated subassemblages also included information about the seasonality of G. lamblia assemblages. Studies with a larger number of samples studied, with only one assemblage A being detected, precludes investigating the differences between assemblage A and B in relation to symptomatic, malnutrition and socio-demographic conditions. Despite the limitations mentioned above, we believe that an important contribution was made through the identification of G. lamblia assemblages circulating in children attending the Bengo General Hospital, Angola.

Future investigations are needed to clarify the current genetic diversity and better understand the importance of local G. lamblia assemblages. Studies with a larger number of samples, also including information about the seasonality of G.
Table 1. *Giardia lamblia* assemblages identified in children with diarrhoea attending the Bengo General Hospital, Angola (N=12) and respective descriptions of sample collection (month/year), gender (male/female), age in months, reported symptomatology (vomiting, fever and lethargy), type of enteric infection identified according to the enteric pathogens detected (simple: the child was infected only by *G. lamblia*; multiple: the child was infected by *G. lamblia* and other enteric pathogens), laboratory results of microscopy and rapid test detection methods performed.

| Identification | Sociodemographic characterization | Symptomatology | Enteric infection | Diagnosis of *Giardia lamblia* | Molecular assemblages |
|---------------|----------------------------------|----------------|------------------|-------------------------------|----------------------|
| Number        | Collection date (month, year)    | Gender | Age (months) | Vomiting | Fever | Lethargy | Type   | Microscopy | Rapid test | ssu | GenBank accession nos. | bg | GenBank accession nos. |
| 1             | February 2013                   | Male   | 11.4          | Yes       | Yes   | No       | Multiple | Negative   | Positive   | B   | MF479750          | n.a. |
| 2             | April 2013                      | Female | 10.5          | Yes       | Yes   | Yes      | Simple   | Negative   | Positive   | A   | MF479751          | A3  | MF565378          |
| 3             | May 2013                        | Female | 8.6           | No        | Yes   | Yes      | Multiple | Negative   | Positive   | B   | MF479752          | B*  | MF565379          |
| 4             | May 2013                        | Male   | 11.3          | Yes       | Yes   | No       | Multiple | Negative   | Positive   | B   | MF479753          | B*  | MF565380          |
| 5             | May 2013                        | Female | 13.1          | No        | Yes   | No       | Multiple | Negative   | Positive   | B   | MF479754          | n.a. |               |
| 6             | June 2013                       | Female | 12.8          | No        | Yes   | Yes      | Multiple | Positive   | Positive   | B   | MF479755          | n.a. |               |
| 7             | August 2013                     | Male   | 9.3           | No        | Yes   | No       | Multiple | Positive   | Positive   | B   | MF479756          | n.a. |               |
| 8             | August 2013                     | Female | 27.4          | No        | No    | No       | Simple   | Positive   | Positive   | B   | MF479757          | B*  | MF565381          |
| 9             | August 2013                     | Female | 14.2          | No        | No    | No       | Multiple | Positive   | Positive   | B   | MF479758          | n.a. |               |
| 10            | August 2013                     | Male   | 27.3          | Yes       | Yes   | No       | Multiple | Positive   | Positive   | B   | MF479759          | n.a. |               |
| 11            | October 2013                    | Female | 30.9          | No        | Yes   | Yes      | Simple   | Positive   | Positive   | B   | MF479760          | n.a. |               |
| 12            | December 2013                   | Male   | 17.3          | No        | Yes   | No       | Multiple | Positive   | Positive   | B   | MF479761          | n.a. |               |

*Subassemblage determination not possible.

n.a.: not applicable.
Authors’ contributions: CG participated throughout the entire sampling, carried out parasitological analysis (microscopy and immunochromatographic rapid tests), contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. FSF carried out molecular analysis, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. ACM carried out parasitological analysis (microscopy and immunochromatographic rapid tests), drafted the initial manuscript as submitted. MCM carried out parasitological analysis (microscopy and immunochromatographic rapid tests) and DNA extraction, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. MB conceptualized and designed the study, coordinated the planning phase of the study, contributed to the analysis and interpretation of data, critically reviewed the manuscript and approved the final manuscript as submitted. SVN conceptualized and designed the study, coordinated the planning phase of the study, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. ASR carried out parasitological analysis (microscopy and immunochromatographic rapid tests) and DNA extraction, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. DPC carried out molecular analysis, critically reviewed the manuscript and approved the final submitted manuscript. MB conceptualized and designed the study, coordinated and supervised data collection, contributed to the analysis and interpretation of data, drafted the initial manuscript and critically reviewed and approved the final submitted manuscript.

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Competing interests: None declared.

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References
1 Monis PT, Andrews RH, Mayrhofer G et al. Genetic diversity within the morphological species Giardia intestinalis and its relationship to host origin. Infect Genet Evol. 2003;3(1):29–38.
2 Lane S, Lloyd D. Current trends in research into the waterborne parasite Giardia. Crit Rev Microbiol. 2002;28(2):123–47.
3 Cacciò SM, Ryan U. Molecular epidemiology of giardiasis. Mol Biochem Parasitol. 2008;160(2):75–80.
4 World Health Organization. The world health report. Geneva: World Health Organization, 1996.
5 Savioli L, Smith H, Thompson A. Giardia and Cryptosporidium join the ‘Neglected Diseases Initiative’. Trends Parasitol. 2006;22(5):203–8.
6 Feng Y, Xiao L. Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. Clin Microbiol Rev. 2011;24(1):110–40.
7 Berkman DS, Lescano AG, Gilman RH et al. Effects of stunning, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet. 2002;359(9306):564–71.
8 Simsek Z, Zeyrek FY, Kurcer MA. Effect of Giardia infection on growth and psychomotor development of children aged 0–5 years. J Trop Pediatr. 2004;50(2):90–3.
9 Carvalho-Costa FA, Gonçalves AQ, Lassance SL et al. Giardia lamblia and other intestinal parasitic infections and their relationships with nutritional status in children in Brazilian Amazon. Rev Inst Med Trop Sao Paulo. 2007;49(3):147–53.
10 Nemtjan J, Ghelamrezaeezhad A, Nemtian E. Giardiasis and other intestinal parasitic infections in relation to anthropometric indicators of malnutrition: a large, population-based survey of schoolchildren in Tehran. Ann Trop Med Parasitol. 2008;102(3):209–14.
11 Botero-Garcés JH, García-Montoya GM, Grisales-Patino D et al. Giardia intestinalis and nutritional status in children participating in the complementary nutrition program, Antioquia, Colombia, May to October 2006. Rev Inst Med Trop Sao Paulo. 2009;51(3):155–62.
12 Ignatius R, Gahutu JB, Klotz C et al. High prevalence of Giardia duodenalis assemblage B infection and association with underweight in Rwandan children. PLoS Negl Trop Dis. 2012;6(6):e1677.
13 Ghoshal U, Shukla R, Pant P et al. Frequency, diagnostic performance of coproantigen detection and genotyping of the Giardia among patients referred to a multi-level teaching hospital in northern India. Pathog Glob Health. 2016;110(7–8):316–20.
14 Sprong H, Cacciò SM, van der Giessen JW. Identification of zoonotic genotypes of Giardia duodenalis. PLoS Negl Trop Dis. 2009;3(12):e558.
15 Lasek-Nesselquist E, Welsh DM, Sogin ML. The identification of a new Giardia duodenalis assemblage in marine vertebrates and a preliminary analysis of G. duodenalis population biology in marine systems. Int J Parasitol. 2010;40(9):1063–74.
16 Zahedi A, Field D, Ryan U. Molecular typing of Giardia duodenalis in humans in Queensland—first report of assemblage E. Parasitology. 2017;144(9):1154–61.
17 Tungtrongchit A, Sookrung N, Indrawattana N et al. Giardia intestinalis in Thailand: identification of genotypes. J Health Popul Nutr. 2010;28(1):42–52.
18 Thompson RC. The zoonotic significance and molecular epidemiology of Giardia and giardiasis. Vet Parasitol. 2004;126(1–2):15–35.
19 Cacciò SM, Thompson RC, McLauchlin J et al. Unravelling Cryptosporidium and Giardia epidemiology. Trends Parasitol. 2005;21(9):430–7.
20 Robertson LJ, Hanevik K, Escobedo AA et al. Giardiasis—why do the symptoms sometimes never stop? Trends Parasitol. 2010;26(2):75–82.
21 Almeida A, Poizio E, Cacciò SM. Genotyping of Giardia duodenalis cysts by new real-time PCR assays for detection of mixed infections in human samples. Appl Environ Microbiol. 2010;76(6):1895–901.
22 Laishtam S, Kang G, Ajijumpur SS. Giardiasis: a review on assemblage distribution and epidemiology in India. Indian J Gastroenterol. 2012;31(1):3–12.
23 Homan WL, Manik TG. Human giardiasis: genotype linked differences in clinical symptomatology. Int J Parasitol. 2001;31(8):822–6.

24 Gelayew T, Lalle M, Hailu A et al. Molecular characterization of human isolates of Giardia duodenalis from Ethiopia. Acta Trop. 2007;102(2):92–9.

25 Pelayo L, Nunez FA, Rojas L et al. Giardia infections in Cuban children: the genotypes circulating in a rural population. Ann Trop Med Parasitol. 2008;102(7):585–95.

26 Al-Mohammed HI. Genotypes of Giardia intestinalis clinical isolates of gastrointestinal symptomatic and asymptomatic Saudi children. Parasitol Res. 2011;108(6):1375–81.

27 Plutzer J, Ongerth J, Karanis P. Giardia taxonomy, phylogeny and epidemiology: facts and open questions. Int J Hyg Environ Health. 2010;213(5):321–33.

28 Tomlinson M, Adams V, Chopra M et al. Survey of iodine deficiency and intestinal parasitic infections in school-going children: Bie Province, Angola. Public Health Nutr. 2010;13(9):1314–8.

29 Sousa-Figueiredo JC, Gamboa D, Pedro JM et al. Epidemiology of malaria, schistosomiasis, geohelminthes, anemia and malnutrition in the context of a demographic surveillance system in northern Angola. PLoS One. 2012;7(4):e33189.

30 Soares Magalhaes RJ, Fancony C, Gamboa D et al. Extending helminth control beyond STH and schistosomiasis: the case of human hemoeneploiasis. PLoS Negl Trop Dis. 2013;7(10):e2321.

31 Oliveira D, Ferreira FS, Atouguia J et al. Infection by intestinal parasites, stunting and anemia in school-aged children from southern Angola. PLoS One. 2015;10(9):e0137327.

32 Gasparinho C, Mirante MC, Centeno-Lima S et al. Etiology of diarrhea due to Giardia duodenalis in children younger than 5 years attending the Bengo General Hospital in Angola. Pediatr Infect Dis J. 2016;35(2):e28–34.

33 Yang H, de Onis M. Algorithms for converting estimates of child malnutrition based on the NCHS reference into estimates based on the WHO Child Growth Standards. BMC Pediatr. 2008;8:19.

34 Hopkins RM, Meloni BP, Groth DM et al. Ribosomal RNA sequencing reveals differences between the genotypes of Giardia isolates recovered from humans and dogs living in the same locality. J Parasitol. 1997;83(1):44–51.

35 Read C, Walters J, Robertson ID et al. Correlation between Giardia duodenalis and diarrhoea. Int J Parasitol. 2002;32(2):229–31.

36 Caccio SM, Di Giacomo M, Pozio E. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype Giardia duodenalis cysts from human faecal samples. Int J Parasitol. 2002;32(8):1023–30.

37 Lalle M, Jimenez-Cardosa E, Caccio SM et al. Genotyping of Giardia duodenalis from humans and dogs from Mexico using a β-giardin nested polymerase chain reaction assay. J Parasitol. 2005;91(1):203–5.

38 Eligio-Garcia L, Cortes-Campos A, Cota-Guajardo S et al. Frequency of Giardia intestinalis assemblages isolated from dogs and humans in a community from Culiacan, Sinaloa, Mexico using β-giardin restriction gene. Vet Parasitol. 2008;156(3–4):205–9.

39 Lebbad M, Ankarklev J, Tellez A et al. Dominance of Giardia assemblage B in Leon, Nicaragua. Acta Trop. 2008;106(1):44–53.

40 Minvielle MC, Molina NB, Polverino D et al. First genotyping of Giardia lamblia from human and animal feces in Argentina, South America. Mem Inst Oswaldo Cruz. 2008;103(1):98–103.

41 Bertrand I, Albertini L, Schwartzbrod J. Comparison of two target genes for detection and genotyping of Giardia lamblia in human feces by PCR and PCR-restriction fragment length polymorphism. J Clin Microbiol. 2005;43(12):5940–4.

42 Ajampur SS, Sankaran P, Kannan A et al. Giardia duodenalis assemblages associated with diarrhea in children in South India identified by PCR-RFLP. Am J Trop Med Hyg. 2009;80(1):16–9.

43 Geurden T, Levecke B, Caccio SM et al. Multilocus genotyping of Cryptosporidium and Giardia in non-outbreak related cases of diarrhoea in human patients in Belgium. Parasitology. 2009;136(10):1161–8.

44 Foronda P, Bargues MD, Abreu-Acosta N et al. Identification of genotypes of Giardia intestinalis of human isolates in Egypt. Parasitol Res. 2008;103(5):1177–81.

45 El Fatni C, Olmo F, El Fatni H et al. First genotyping of Giardia duodenalis and prevalence of enteroparasites in children from Tetouan (Morocco). Parasite. 2014;21:48.

46 Xiao L, Fayer R. Molecular characterisation of species and genotypes of Cryptosporidium and Giardia and assessment of zoonotic transmission. Int J Parasitol. 2008;38(11):1239–55.

47 Kohli A, Bushen OY, Pinkerton RC et al. Giardia duodenalis assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg. 2008;102(7):718–25.

48 Ryan U, Caccio SM. Zoonotic potential of Giardia. Int J Parasitol. 2013;43(12–13):943–56.

49 Wielinga C, Ryan U, Andrew Thompson RC et al. Multi-locus analysis of Giardia duodenalis intra-assemblage B substitution patterns in cloned culture isolates suggests sub-assemblage B analyses will require multi-locus genotyping with conserved and variable genes. Int J Parasitol. 2011;41(5):495–503.

50 Caccio SM, Beck R, Lalle M et al. Multilocus genotyping of Giardia duodenalis reveals striking differences between assemblages A and B. Int J Parasitol. 2008;38(13):1523–31.

51 Ferreira FS, Centeno-Lima S, Gomes J et al. Molecular characterization of Giardia duodenalis in children from the Cufada Lagoon Natural Park, Guinea-Bissau. Parasitol Res. 2012;111(5):2173–7.

52 Farthing MJ. Giardiasis. Gastroenterol Clin North Am. 1996;25(3):493–515.

53 Ortega YR, Adam RD. Giardia: overview and update. Clin Infect Dis. 1997;25(3):545–9; quiz 550.