Published in final edited form as:

J Microbiol Methods. 2017 October ; 141: 35–41. doi:10.1016/j.mimet.2017.07.004.

Stool antigen immunodetection for diagnosis of *Giardia duodenalis* infection in human subjects with HIV and cancer

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Abstract

Human infection with the protozoan parasite *Giardia duodenalis* is one the most common parasitic diseases worldwide. Higher incidence rates of giardiasis have been reported from human subjects with multiple debilitating chronic conditions, including hypogammaglobulinemia and common variable immunodeficiency (CVID). In the current study, stool specimens were collected from 199 individuals diagnosed with HIV or cancer and immunocompetent subjects. The sensitivity of microscopy-based detection on fresh stool preparations, trichrome staining and stool antigen immunodetection for the diagnosis of *G. duodenalis* were 36%, 45.5% and 100%, respectively when compared with a highly sensitive stool-based PCR method as the gold standard. Further multilocus molecular analyses using glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) demonstrated that the AI genotype of *G. duodenalis* was the most prevalent,

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Author contribution

M.N. collected clinical samples and performed most of the experiments. B. K.-K., I.S., H.K., N.P.M.L., L.A.L., M.F.K., and S.M.S. contributed hypotheses, analyzed the data and critically read the manuscript. Z.B. conceived the study, contributed hypotheses, designed the experiments, performed some of the experiments, and analyzed the data. S.S.-M. conceived the study, contributed hypotheses, designed the experiments, analyzed the data, and wrote the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

followed by the AII genotype and mixed (AI+B) infections. We concluded that stool antigen immunodetection-based immunoassays and stool-based PCR amplification had comparable sensitivity and specificity for the diagnosis of *G. duodenalis* infections in these populations. Stool antigen detection-based diagnostic modalities are rapid and accurate and may offer alternatives to conventional microscopy and PCR-based diagnostic methods for the diagnosis of *G. duodenalis* in human subjects living with HIV or cancer.

Keywords

Giardia duodenalis; giardiasis; HIV; Cancer; assemblage; genotype

1. Introduction

Human giardiasis is one of the most prevalent enteric protozoan infections globally, with an estimated 280 million cases worldwide per year (Farthing, 1996). The prevalence rates range from 2–5% in the developed world and 20–30% in the developing countries (Yoder et al., 2012). Human giardiasis is considered the most commonly identified parasitic infection in humans in the United States (~10 cases/100,000), with approximately 20,000 cases reported annually (Scallan et al., 2011; Yoder et al., 2012). The etiologic agent, *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*), is an enteric protozoan parasite that colonizes the upper portions of the small intestine of a wide range of mammalian hosts (Farthing, 1996; Adam, 2001). Humans are infected by the ingestion of the quadrinucleated cysts present in contaminated food and water. Symptomatic infections in humans are characterized by a wide range of gastrointestinal manifestations, ranging from abdominal cramps, flatulence, diarrhea and nausea to a malnutrition syndrome and/or stunted growth, especially in children (Berkman et al., 2002; Veenemans et al., 2011). *G. duodenalis* consists of a genetically heterogeneous group that has been clustered into 8 clades, referred to as assemblages A–H, based on sequences of the 18S rRNA and housekeeping genes such as triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*) and β -giardin (*bg*) (Lebbad et al., 2008; Monis et al., 2009).

It has been suggested that increased incidence rates of *G. duodenalis* infections are associated with certain immunodeficiency conditions, including Bruton's X-linked agammaglobulinemia and common variable immunodeficiency (CVID) (Perlmutter et al., 1985; Furtado et al., 2012). However, there are contradictory reports as to whether HIV infection or cancer may predispose human subjects to greater risk of *G. duodenalis* infection. While several studies demonstrated significantly increased rates of *G. duodenalis* infection in individuals living with HIV (Feitosa et al., 2001; Sanyaolu et al., 2011), others failed to provide a link between increased incident rates of *G. duodenalis* infection in HIV-positive individuals (Escobedo et al., 2014). Furthermore, limited evidence is available about the distribution of *G. duodenalis* assemblages/genotypes in individuals living with HIV or cancer (Lim et al., 2011). Preliminary studies suggested a positive correlation between infection with a given *G. duodenalis* assemblage and HIV infection. While Lim et al. showed that all HIV-positive subjects examined were infected with assemblage A of *G. duodenalis*

(2011), Matey et al found a direct association between HIV infection status and infection with assemblage B, but not assemblage A, of *G. duodenalis* (Matey et al., 2016).

Microscopy-based diagnostic modalities, whether on fresh samples or stained slides are still the most commonly-used methods for the diagnosis of infection with *G. duodenalis*, especially in resource-limited settings (McHardy et al., 2014; Silva et al., 2016). However, several lines of experimental evidence have suggested that microscopy-based diagnostic methods are labor-intensive and show limited sensitivity compared with highly sensitive and specific methods such as the stool-based PCR assays (Koehler et al., 2014). In recent years, stool-based PCR methods have been developed to detect infections with *G. duodenalis* in a wide range of clinical settings (Calderaro et al., 2010; Becker et al., 2015). These assays are considered gold standards for the detection of several enteric protozoal infections, including *G. duodenalis* (Calderaro et al., 2010; Becker et al., 2015; Aguiar et al., 2015). Despite enhanced sensitivity, stool-based PCR assays require specialized equipment, well-equipped diagnostic laboratories, specialized training and skilled staff. These disadvantages combined with increased rates of false positives render PCR-based assays of limited feasibility for less-equipped diagnostic laboratories, especially in developing countries where human giardiasis is endemic (Elsafi et al., 2013).

Stool antigen immunodetection-based assays have increasingly been used in recent years for the detection of *G. duodenalis* infections in humans in a wide range of clinical settings in underdeveloped and developing countries (Christy et al., 2012; Alexander et al., 2013). The stool antigen immunodetection-based assays have been used in routine diagnostic laboratories because of their demonstrated higher sensitivity compared with conventional microscopy-based diagnostic modalities (Strand et al., 2008; Alexander et al., 2013). These assays are rapid and convenient and possess comparable sensitivity and specificity to those of stool-based PCR methods for the diagnosis of *G. duodenalis* infections in humans (Ng et al., 2005; Schuurman et al., 2007).

The aim of the current study, therefore, was to determine the occurrence of *G. duodenalis* in individuals living with HIV or cancer and to characterize *G. duodenalis* assemblages/genotypes occurring among these populations. We also aimed to evaluate the sensitivity and specificity of a stool antigen immunodetection-based assay in comparison with conventional microscopy and stool-based PCR modalities for the diagnosis of *G. duodenalis* infection in the populations studied.

2. Materials and Methods

2.1. Ethical Approval

Informed consent was obtained from all individual participants included in the study. This study was approved by the Ethical Committee of Kerman University of Medical Sciences and the Iranian Ministry of Health, Treatment and Medical Training Protection Code of Human Subjects in Medical Research.

2.2. Study subjects

Fecal specimens included in this study were collected from human subjects with HIV infection ($n = 72$), subjects diagnosed with cancer ($n = 59$) or immunocompetent controls ($n = 68$) from Kerman, central Iran. Human enteric parasites are frequently reported from this area, and it has been reported that up to 47% of residents are infected with *G. duodenalis* (Zia-Ali and Massoud, 1997).

2.3. CD4⁺ T cell count

Three to five milliliters of peripheral blood were collected in Mediplus EDTA tubes (Sunphoria Co. Ltd., Taiwan) and 20 μ l of patients' blood was stained with a fluorescent anti-CD4 monoclonal antibody using a Partec CD4 Easy Count kit (Partec GmbH, Münster, Germany) following manufacturers' instructions. The CD4⁺ T cell absolute count was performed using a Partec CyFlow[®] Counter as described before (Zijenah et al., 2006; Fryland et al., 2006).

2.4. Stool specimens and processing

Fecal samples (2–5 grams) were collected aseptically from the subjects, transported immediately to the laboratory, aliquoted and kept at -20°C until further analysis.

2.5. Stool microscopy on wet mount preparations

Trophozoites and/or cysts of *G. duodenalis* in fresh feces were identified by light microscopy on wet preparations stained with Lugol's iodine solution as described elsewhere (Thornton et al., 1983). The Lugol's iodine solution stains *G. duodenalis* nuclei yellow to brown and the cytoplasm is stained yellow, resulting in enhanced parasite visibility in wet mount preparations. However, the Lugol's iodine solution is toxic to trophozoites and consequently excludes the possibility of observing motile trophozoites in iodine-stained wet preparations (Wolfe, 1992).

2.6. Trichrome staining for the detection of *G. duodenalis* trophozoites/cysts

Two smears were prepared from fresh stools on glass slides, followed by immediate fixation in Bouin's fixative solution (saturated picric acid-40% formalin-glacial acetic acid; 73:25:2) for 20 min. The smears subsequently were rinsed in 70% alcohol for at least 1 min and then stained with trichrome for 10–20 min as described earlier elsewhere (Nace et al., 1999). The slides were subsequently rinsed in acidified alcohol (1% glacial acetic acid in 96% ethanol), followed by dehydration in 90% alcohol and absolute ethanol. Finally, the slides were placed in xylene for another 5 min, covered by a coverslip and examined under the microscope ($\times 400$). The *G. duodenalis* parasites are stained green to red with trichrome and the internal organelles are visible as reddish/brown structures in a greenish background.

2.7. Stool antigen immunodetection of *G. duodenalis*

Stool samples from human subjects were diluted using the kit diluent and were subsequently centrifuged at $800\times g$ for 10 min. *G. duodenalis* antigens were detected in stool samples using a monoclonal antibody-based immunodetection test (Catalog number 7069; Biomerica Inc., Hannover, Germany) according to the manufacturer's instructions. This kit is an

ELISA-based stool antigen immunodetection assay and is intended to specifically and qualitatively detect *Giardia* antigens in the stool in a wide range of clinical settings. The well strips were measured in an automatic microtiter plate reader (BioTek ELX50, Winooski, Vermont, USA) at 450 nm. Stool specimens with an optical density of 0.05 or greater after subtraction of the negative control optical density were considered as positive.

2.8. Genomic DNA (gDNA) extraction

The trophozoites or cysts walls of *G. duodenalis* were mechanically disrupted using glass beads (0.45–0.52 mm diameter), and lysed by vortexing as well as by repeated freezing and thawing of the cysts/trophozoites in liquid nitrogen as previously described (Babaei et al., 2011). After the addition of proteinase K, the suspensions were incubated at 56°C for 4–6 h, followed by gDNA extraction using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentrations and the purity of extracted gDNA samples were measured at 260/280 nm, respectively using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The gDNA samples were stored at –20° C until further analysis.

2.9. Semi-nested PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) genes of *G. duodenalis*

Semi-nested stool-based PCR assay targeting the glutamate dehydrogenase (*gdh*) locus of *G. duodenalis* was performed in a 25 µL volume, containing 12.5 pmol of each forward and reverse primers and 10 µL of 2× Master Mix Red (Ampliqon, Copenhagen, Denmark), containing 1.5 mM

MgCl₂. Primers used were External Forward Primer, GDHeF (5'-TCA ACG TYA AYC GYG GYT TCC GT-3'), Internal Forward Primer, GDHiF (5'- CAG TAC AAC TCY GCT CTC GG-3') and Reverse Primer, GDHiR (5'-GTT RTC CTT GCA CAT CTC C-3') to specifically amplify a 432-bp fragment as described earlier (Read et al., 2004). The amplification program for the first PCR consisted of 30 cycles of 30 s at 94°C, 20 s at 60°C and 45 s at 72°C, and final extension at 7 min at 72°C using an Analytik Jena thermocycler (Analytik Jena, Jena, Germany). In the second PCR, amplification consisted of 35 cycles, and the annealing temperature was increased to 62°C and other parameters were left unchanged.

Ten microliters of the PCR products resulting from the amplification of the *gdh* locus of *G. duodenalis* were digested with 5 U of *Bsp*LI (*Nla*IV; Fermentas, St. Leon-Rot, Germany) in 1× Tango buffer for 2 h at 37°C in order to distinguish between assemblages A (AI/AII) and B as described before (Amar et al., 2002; Babaei et al., 2016).

The semi-nested stool-based PCR assay targeting the *tpi* locus of *G. duodenalis* was performed as described earlier (Babaei et al., 2016).

The PCR products were separated on 1% (undigested products) or 3% (digestion products) agarose gel (Takara Shuzo, Kyoto, Japan) in Tris-borate-EDTA buffer, stained with ethidium bromide (0.1 mg/mL), and visualized under UV light (UVItect, Cambridge, UK).

2.10. Statistical analyses

Data were analyzed using GraphPad Prism 6.07 software (GraphPad, San Diego, CA) and expressed as the mean $\pm$ SEM. For statistical analyses, a two-tailed Mann-Whitney *U* test was used, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study population

We carried out a cross-sectional observational study, recruiting 68 immunocompetent controls aged 7–72 year-old (33.31 ± 2.08), 72 individuals living with HIV aged 11–61 year-old (39.99 ± 1.12), and 59 individuals diagnosed with cancer from an age of 9 months to 74 years (39.32 ± 2.67). The age of human subjects infected with *G. duodenalis* ($n = 11$) ranged from 6 to 53 year-old (30.73 ± 4.69). The demographic characteristics of human subjects included in this study as well as human subjects with *G. duodenalis* are presented in Tables 1 and 2, respectively.

3.2. CD4⁺ T cell counts in human subjects concurrently infected with enteric parasites is significantly lower than those without concurrent enteric parasitic infections

As shown in Figure 1, our analyses showed that HIV-positive subjects infected with at least one enteric parasite species had significantly lower CD4⁺ T cells/ μ L ($p = 0.02$) compared with those with no enteric parasitic infections. These parasites included *G. duodenalis*, *Blastocystis* spp., *Entamoeba coli*, *Cryptosporidium* spp., *Hymenolepis nana*, and *Cyclospora* sp. The CD4⁺ T cell counts in HIV-positive subjects with concomitant enteric parasitic infections ranged from 87–555 cells/ μ L (277.5 ± 31.34), whereas the CD4⁺ T cell counts in HIV-positive subjects without concomitant enteric parasitic infections ranged from 61–1050 cells/ μ L (459.3 ± 38.67). However, the CD4⁺ T cell counts in HIV-positive individuals co-infected with *G. duodenalis* ($n = 4$) ranged from 250–377 cells/ μ L.

3.3. Stool antigen immunodetection of *G. duodenalis* is as sensitive and specific as stool- based PCR assays

The overall prevalence rates of *G. duodenalis* infection among the study population by microscopy on fresh stool preparations or slides stained with trichrome, stool-based antigen immunodetection assay, and direct stool-based PCR amplification 2.01% (4/199), 2.51% (5/199), 5.52% (11/199), and 5.52% (11/199), respectively. Using a stool-based semi-nested PCR, we showed that 3/68 (4.41%), 4/72 (5.55%) and 4/59 (6.77%) of immunocompetent controls, HIV-positive and cancer subjects were positive for *G. duodenalis*, respectively. There was a 100% correlation between the results obtained from the stool antigen-based immunodetection method and those from a highly sensitive semi-nested stool-based PCR assay. The sensitivity of microcopy-based detection on fresh stool preparations, trichrome staining and stool antigen immunodetection for the diagnosis of *G. duodenalis* were 36%, 45.5% and 100% when compared with a highly sensitive stool-based PCR method as the gold standard. However, all of these three methods had a specificity of 100% for the diagnosis of *G. duodenalis* in the stool specimens of human subjects examined.

Altogether these findings suggest that the occurrence of *G. duodenalis* in individuals with HIV and with cancer were not significantly different from those of immunocompetent controls, and that stool antigen immunodetection assays showed comparable results to highly sensitive PCR-based assays in the diagnosis of *G. duodenalis*.

3.4. Characterization of *G. duodenalis* clinical isolates based on the PCR-RFLP of the *gdh* and *tpi* loci

A 432-bp fragment within the *gdh* locus of *G. duodenalis* was amplified directly from stools of human subjects using primers GDHeF, GDHiF and GDHiR as described before (Babaei et al., 2011; Babaei et al., 2016). The PCR amplicons were the same size for both assemblages A and B parasites (Figure 2A). Our analyses showed that 11/199 (5.52%) human subjects were positive for *G. duodenalis* by direct PCR amplification from the stool. We found that 3/68 (4.41%), 4/72 (5.55%) and 4/59 (6.77%) of immunocompetent controls, HIV-positive subjects and human subjects with cancer were, respectively, stool-positive for *G. duodenalis* by using direct PCR amplification from the stool. After digestion with the restriction enzyme *Bsp*LI (*Nla*IV), the expected lengths of the amplified fragments were 90-, 120-, and 150bp for the AI genotype and 70-, 80-, 90-, 120-bp for the AII genotype (Figure 2B). The *Bsp*LI digestion of the PCR products resulting from the *gdh* locus amplification resulted in two bands of 120- and 290-bp for assemblage B of *G. duodenalis*.

A 476-bp (assemblage A) and a 140-bp (assemblage B) fragment within the *tpi* locus of *G. duodenalis* were amplified using specific primers as described earlier (Babaei et al., 2016). The subsequent digestion of the PCR products with the restriction enzyme *Rsa*I resulted in fragments of 437- and 15-bp for the AI genotype and 235-, 232-, and 15-bp for the AII genotype (Figure 2C).

Our analyses using the *gdh* locus showed that 6/11 (54.54%) and 3/11 (27.27%) human subjects were singly infected with AI and AII genotypes of *G. duodenalis*, respectively, whereas mixed infections (AI+B) were found in 2/11 (18.19%) human subjects examined. Molecular analyses using the *tpi* locus demonstrated that 5/11 (45.45%) and 4/11 (36.36%) human subjects were singly infected with AI and AII genotypes of *G. duodenalis*, respectively. Mixed infections (AI+B) were observed in 2 (18.9%) patients. No mixed infections at intra-genotype levels (AI+AII) were detected. Table 2 represents the assemblages/genotypes of *G. duodenalis* identified in the human subjects studied.

4. Discussion

This study examined the incidence and the assemblages/genotypes of *G. duodenalis* found in HIV positive subjects, human subjects with cancer and immunocompetent controls. We also evaluated four diagnostic modalities for the detection of *G. duodenalis* in the human subjects studied. The most important findings of these studies were that (i) HIV-positive subjects with concomitant enteric parasitic infections had significantly lower CD4⁺ T cell counts as compared with HIV positive subjects without any concomitant enteric parasitic infections; (ii) A stool antigen immunodetection-based modality and a highly sensitive stool-based PCR method had comparable sensitivity and specificity and that the conventional microscopy-based diagnostic methods, either on fresh stool specimens or stained with permanent stains,

had lower sensitivity for the detection of *G. duodenalis* infections; and (iii) HIV-positive subjects, human subjects with cancer and immunocompetent controls were equally at the risk of acquiring infection with *G. duodenalis*, and that no increased prevalence rates of *G. duodenalis* infection were found among the three groups. HIV-positive human subjects with low CD4⁺ T cell counts are at higher risk of acquiring a variety of enteric parasite infection, including *G. duodenalis* infection (Angarano et al., 1997; Mengist et al., 2015). It is well-known that CD4⁺ T cells play protective roles against *G. duodenalis* infection via the production of multiple cytokines (e.g. IFN- γ , IL-17A) required for defense against *G. duodenalis* infection in murine models of giardiasis (Mengist et al., 2015; Solaymani-Mohammadi and Singer, 2010; Solaymani-Mohammadi and Singer, 2011; Dann et al., 2015). As such, Angarano et al (1997) showed that HIV-positive human subjects with low CD4⁺ T cell counts had greater risk of *G. duodenalis* acquisition as compared with those with higher CD4⁺ T cell counts. These findings highlight important roles played by CD4⁺ T cells during *G. duodenalis* infections in mice and humans.

In the current study, we first compared peripheral CD4⁺ T cell counts in HIV-positive human subjects with or without at least one enteric parasitic infection. These parasites included *G. duodenalis*, *Blastocystis* spp., *Cryptosporidium* spp., *Hymenolepis nana*, *Cyclospora* sp., and the non-pathogenic *Entamoeba coli*. We demonstrated that HIV-positive subjects with at least one enteric parasitic infection had significantly lower CD4⁺ T cell counts compared with those with no detectable enteric parasitic infections (Figure 1). However, limitations of the sample size in the current study precluded a comparison between CD4⁺ T cell counts in HIV-positive human subjects in the absence or the presence of *G. duodenalis* infection. It is still unclear whether HIV-positive human subjects with decreased CD4⁺ counts are at higher risk of *G. duodenalis* acquisition or decreased CD4⁺ T cell counts are the direct consequences of HIV-enteric parasitic co-infection. Nonetheless, the causality of decreased CD4⁺ T cell counts in human subjects with HIV-enteric parasitic coinfections as well as the direction of the causality warrant more detailed scrutiny in future case control studies.

High incidence rates of *G. duodenalis* infection have been reported in HIV-positive subjects in all geographical regions (Farthing, 1997; Sanyaolu et al., 2011), especially in those with decreased CD4⁺ T cell counts (Angarano et al., 1997), although several studies failed to find a correlation between HIV infection and high rates of *G. duodenalis* infection (Escobedo et al., 2014). We tested the incidence rates of *G. duodenalis* in HIV-positive subjects and human subjects with cancer in comparison with immunocompetent controls. We also characterized the assemblages/genotypes found among these subjects by a PCR-RFLP method. By targeting the *gdh* and *tpi* loci of the *G. duodenalis* parasites identified in the current study, we demonstrated that HIV-positive subjects and immunocompetent controls had comparable incidence rates of *G. duodenalis* infection. Further analysis showed that human subjects positive for *G. duodenalis* in the current study were either singly (AI, AII) or dually (AI+B) infected with *G. duodenalis* assemblage A. No mixed infections at intra-genotype levels (AI+AII) were detected. Collectively, these findings are consistent with our previous observations that *G. duodenalis* assemblage A (AI) was the dominant parasite found in the Kerman area of central Iran (Babaei et al., 2016).

Several studies have investigated the propensity of genetically-diverse *Giardia* isolates to induce symptomatic disease in immunocompetent controls; it has been suggested that some given assemblages/genotypes are associated with symptomatic disease, whereas others are not (Babaei et al., 2016; Solaymani-Mohammadi and Singer, 2011; Solaymani-Mohammadi and Singer, 2013). These studies have reported conflicting results as to the association of a given assemblage/genotype of *G. duodenalis* and symptomatic disease and while some studies have found a correlation between *G. duodenalis* assemblage B and HIV infection (Matey et al., 2016), others have found no such a link (Escobedo et al., 2014). In the current study, both assemblages A and B were found in HIV-positive subjects, whereas human subjects with cancer were only infected with *G. duodenalis* assemblage A (AI, AII). However, it still remains to be determined whether a given assemblage/genotype is more prevalent in HIV-positive subjects and whether a given assemblage/genotype is associated with symptomatic disease in HIV-positive subjects. However, the limitations of the sample size in the current study precluded a definite conclusion. Increased susceptibility of human subjects with cancer to enteric parasitic infections has been documented (Nevez et al., 2015). The enteric nematode, *Strongyloides stercoralis* can cause a hyperinfection syndrome in human subjects with multiple types of cancer by spreading to extra-intestinal sites, including the mesenteric lymph nodes, liver, the lungs, pancreas, kidney and the brain (Grove, 1996; Kinjo et al., 2015). In the current study, *G. duodenalis* assemblage A was found in all human subjects with cancer (Table 2). Several sporadic cases of *G. duodenalis* infection have been reported in pediatric and adult human subjects with hematologic malignancy or solid tumors (Furukawa et al., 2011). However, comprehensive studies investigating the prevalence of *G. duodenalis* in human subjects with cancer are scarce. In the current study, we found that 4/59 of human subjects with cancer had *G. duodenalis* infection, whereas 3/68 of immunocompetent controls were detected to have *G. duodenalis* infection (Table 2). These findings again demonstrated that human subjects with cancer had comparable incidence rates of *G. duodenalis* acquisition to immunocompetent controls. Our further analysis also demonstrated that *G. duodenalis* assemblage A (AI, AII) was the only assemblage found in human subjects with cancer (Table 2).

In the current study, we found that all three test groups had comparable incidence rates of *G. duodenalis* infections. The *G. duodenalis* assemblage A was the dominant parasite found in these populations irrespective of subjects' clinical status. These findings suggest that individuals living with HIV and cancer are not likely to be at greater risk for *G. duodenalis* acquisition as compared with immunocompetent controls. However, our findings could have been confounded by the fact that the subjects enrolled in the current study had access to intensive health care facilities on a regular basis, resulting in reduced *G. duodenalis* infection rates.

Conventional microscopy-based methods are still the most commonly-used diagnostic modalities in clinical parasitology laboratories (Ryan et al., 2017). In spite of the simplicity of the conventional microscopy-based methods for the detection of *G. duodenalis* infection, these methods require the observation of intact cysts or trophozoites in fecal specimens and, therefore, the degenerated cysts/trophozoites may not be detected. Furthermore, the number of cysts in chronic cases are extremely low and cysts are excreted intermittently, therefore, the conventional microscopy-based modalities can only detect up to one-third of chronic

infections when performed on a single fecal specimen (McHardy et al., 2014). Three consecutive stool microcopy examinations can detect up to 90% of infections, but it is considered labor-intensive and not applicable in areas in which human giardiasis is endemic. Additionally, methods such as examining the duodenal fluid aspirates obtained by esophagogastroduodenoscopy (EGD) for trophozoites, entero-test (string test) or biopsy of the small intestine offer more sensitive methods of diagnosis, but are rarely used because they are expensive and invasive in nature (Goka et al., 1990; Oberhuber et al., 2016). Therefore, alternative techniques have been evaluated to overcome limitations of the conventional microscopy-based diagnostic methods in order to achieve more accurate and specific diagnosis. In recent years there has been greater attention given to DNA-based diagnostic methods, including conventional and real-time PCR-based diagnostic modalities (Laude et al., 2016). However, stool-based PCR assays require specialized equipment, well-equipped diagnostic laboratories, specialized training and skilled staff. These drawbacks, combined with higher rates of false positives, make PCR-based assays of limited practicality for less-equipped diagnostic parasitology laboratories, especially in developing countries (Elsafi et al., 2013).

Stool antigen immunodetection-based modalities have been increasingly used for diagnosis of *G. duodenalis*, mainly due to their significantly higher sensitivity and accuracy in detecting infections with the parasite. These techniques require much less time and are considered reliable to test large numbers of clinical samples in a short period of time with high sensitivity and specificity (Christy et al., 2002; Ng et al., 2005; Schuurman et al., 2007). In the current study, we compared the conventional microscopy and an antigen detection method for diagnosis of *G. duodenalis* infections in two patient populations and a healthy control group. Our analysis demonstrated that a stool antigen immunodetection-based modality was both sensitive and specific when employed to detect *G. duodenalis* infection and the results were comparable to those of a sensitive stool-based PCR assay. These results are in agreement with several previous reports, demonstrating that stool antigen immunodetection-based modalities represent rapid, reliable, sensitive, specific and standardized immunoassay test for routine diagnosis of *G. duodenalis* infection in humans (Christy et al., 2012; Ng et al., 2005; Schuurman et al., 2007).

Stool antigen immunodetection protocols can represent appropriate alternatives to traditional methods in most parasitology laboratories, where a large number of tests are performed on a daily basis in order to ensure the accuracy and the reliability of the results. Knowing the actual status and the distribution of *G. duodenalis* assemblages/genotypes among immunocompromised subjects and human subjects with cancer are important measures for planning and decision-making processes towards implementing control strategies. In conclusion, we suggest that at least one of these sensitive diagnostic modalities to be routinely performed in addition to conventional microscopy-based methods to ensure accurate and reliable detection of *G. duodenalis* in immunocompromised subjects in reference laboratories, academic centers and children's hospitals.

Conclusion

Based on these findings, we concluded that stool antigen immunodetection-based immunoassays and stool-based PCR amplification had comparable sensitivity and specificity for the diagnosis of *G. duodenalis* infections in these populations. Stool antigen immunodetection-based diagnostic modalities may offer alternatives to conventional microscopy and PCR for the diagnosis of *G. duodenalis* in individuals living with HIV or cancer.

Acknowledgements.

We thank patients and healthy volunteers who participated in this study. We are grateful to Mohsen Zahedi and Mohammad A. Mohammadi for technical assistance. This work was supported by a grant from the Kerman University of Medical Sciences Vice Chancellor for Research (to ZB, SSM), under contract 94/449. Work in the Singer laboratory is supported by National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID) grant AI109591.

References

1. Adam RD (2001) Biology of *Giardia lamblia*. Clin Microbiol Rev 14, 447–475. [PubMed: 11432808]
2. Aguiar JM, Silva SO, Santos VA, Rossa GA, Taniwaki SA, Gregori F, Keid LB, Soares RM (2015) Multilocus amplification of genomic DNA from single cysts of *Giardia duodenalis* separated using micromanipulation technique. Exp Parasitol 157, 84–87. [PubMed: 26172406]
3. Alexander CL, Niebel M, Jones B (2013) The rapid detection of *Cryptosporidium* and *Giardia* species in clinical stools using the Quik Chek immunoassay. Parasitol Int 62, 552–553. [PubMed: 23981506]
4. Amar CF, Dear PH, Pedraza-Díaz S, Looker N, Linnane E, McLauchlin J (2002) Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. J Clin Microbiol 40, 446–452. [PubMed: 11825955]
5. Angarano G, Maggi P, Di Bari MA, Larocca AM, Congedo P, Di Bari C, Brandonisio O, Chiodo F (1997) Giardiasis in HIV: a possible role in patients with severe immune deficiency. Eur J Epidemiol 13, 485–487. [PubMed: 9258558]
6. Babaei Z, Malihi N, Zia-Ali N, Sharifi I, Mohammadi MA, Kagnoff MF, Eckmann L, Singer SM, Solaymani-Mohammadi S (2016) Adaptive immune response in symptomatic and asymptomatic enteric protozoal infection: evidence for a determining role of parasite genetic heterogeneity in host immunity to human giardiasis. Microbes Infect 18, 687–695. [PubMed: 27401766]
7. Babaei Z, Oormazdi H, Rezaie S, Rezaeian M, Razmjou E (2011) *Giardia intestinalis*: DNA extraction approaches to improve PCR results. Exp Parasitol 128, 159–162. [PubMed: 21315715]
8. Becker SL, Chatigre JK, Gohou JP, Coulibaly JT, Leuppi R, Polman K, Chappuis F, Mertens P, Herrmann M, N'Goran EK, Utzinger J, von Müller L (2015) Combined stoolbased multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire. Clin Microbiol Infect 21, 591e1–10. [PubMed: 25743578]
9. Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM (2002) Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet 359, 564–571. [PubMed: 11867110]
10. Calderaro A, Gorrini C, Montecchini S, Peruzzi S, Piccolo G, Rossi S, Gargiulo F, Manca N, Dettori G, Chezzi C (2010) Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. Diagn Microbiol Infect Dis 66, 261–267. [PubMed: 19903583]
11. Christy NC, Hencke JD, Escueta-De Cadiz A, Nazib F, von Thien H, Yagita K, Ligaba S, Haque R, Nozaki T, Tannich E, Herbein JF, Petri WA, Jr (2012) Multisite performance evaluation of an enzyme-linked immunosorbent assay for detection of *Giardia*, *Cryptosporidium*, and *Entamoeba histolytica* antigens in human stool. J Clin Microbiol 50, 1762–1763. [PubMed: 22378909]

12. Dann SM, Manthey CF, Le C, Miyamoto Y, Gima L, Abraham A, Cao AT, Hanson EM, Kolls JK, Raz E, Cong Y, Eckmann L (2015) IL-17A promotes protective IgA responses and expression of other potential effectors against the lumen-dwelling enteric parasite *Giardia*. *Exp Parasitol* 156, 68–78. [PubMed: 26071205]
13. Elsafi SH, Al-Maqati TN, Hussein MI, Adam AA, Hassan MM, Al Zahrani EM (2013) Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of *Giardia lamblia* and *Cryptosporidium parvum*. *Parasitol Res* 112, 1641–1646. [PubMed: 23411740]
14. Escobedo AA, Almirall P, Alfonso M, Cimerman S, Chacín-Bonilla L (2014) Sexual transmission of giardiasis: a neglected route of spread? *Acta Trop* 132, 106–111. [PubMed: 24434784]
15. Farthing MJ (1997) The molecular pathogenesis of giardiasis. *J Pediatr Gastroenterol Nutr* 24, 79–88. [PubMed: 9093992]
16. Feitosa G, Bandeira AC, Sampaio DP, Badaró R, Brites C (2001) High prevalence of giardiasis and strongyloidiasis among HIV-infected patients in Bahia, Brazil. *Braz J Infect Dis* 5, 339–344. [PubMed: 12010598]
17. Fryland M, Chaillet P, Zachariah R, Barnaba A, Bonte L, Andereassen R, Charrondière S, Teck R, Didakus O (2006) The Partec CyFlow Counter could provide an option for CD4⁺ T-cell monitoring in the context of scaling-up antiretroviral treatment at the district level in Malawi. *Trans R Soc Trop Med Hyg* 100, 980–985. [PubMed: 16542690]
18. Furtado AK, Cabral VL, Santos TN, Mansour E, Nagasako CK, Lorena SL, Pereira-Filho RA (2012) *Giardia* infection: protein-losing enteropathy in an adult with immunodeficiency. *World J Gastroenterol* 18, 2430–2433. [PubMed: 22654437]
19. Furukawa M, Lee L, Ikegami T, Maeda T, Nishiyama K, Itaba S, Funakoshi A (2011) Giardiasis in the pancreas accompanied by pancreatic cancer. *Pancreas* 40:168–169. [PubMed: 21160381]
20. Goka AK, Rolston DD, Mathan VI, Farthing MJ (1990) The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis. *Trans R Soc Trop Med Hyg* 84, 66–67. [PubMed: 2345928]
21. Grove DI (1996) Human strongyloidiasis. *Adv Parasitol* 38, 251–309. [PubMed: 8701797]
22. Kinjo T, Nabeya D, Nakamura H, Haranaga S, Hirata T, Nakamoto T, Atsumi E, Fuchigami T, Aoki Y, Fujita J (2015) Acute respiratory distress syndrome due to *Strongyloides stercoralis* infection in a patient with cervical cancer. *Intern Med* 54, 83–87. [PubMed: 25742900]
23. Koehler AV, Jex AR, Haydon SR, Stevens MA, Gasser RB (2014) *Giardia*/giardiasis—a perspective on diagnostic and analytical tools. *Biotechnol Adv* 32, 280–289. [PubMed: 24189092]
24. Laude A, Valot S, Desoubaux G, Argy N, Nourrisson C, Pomares C, Machouart M, Le Govic Y, Dalle F, Botterel F, Bourgeois N, Cateau E, Leterrier M, Le Pape P, Morio F (2016) Is real-time PCR-based diagnosis similar in performance to routine parasitological examination for the identification of *Giardia intestinalis*, *Cryptosporidium parvum*/*Cryptosporidium hominis* and *Entamoeba histolytica* from stool samples? Evaluation of a new commercial multiplex PCR assay and literature review. *Clin Microbiol Infect* 22, 190e1–8. [PubMed: 26548509]
25. Lebbad M, Ankarklev J, Tellez A, Leiva B, Andersson JO, Svard S (2008) Dominance of *Giardia* assemblage B in Leon, Nicaragua. *Acta Trop* 106, 44–53. [PubMed: 18325480]
26. Lim YA, Iqbal A, Surin J, Sim BL, Jex AR, Nolan MJ, Smith HV, Gasser RB (2011) First genetic classification of *Cryptosporidium* and *Giardia* from HIV/AIDS patients in Malaysia. *Infect Genet Evol* 11, 968–974. [PubMed: 21439404]
27. Matey EJ, Tokoro M, Mizuno T, Matsumura T, Nagamoto T, Bi X, Oyombra JA, Sang WK, Songok EM, Ichimura H (2016) Positive correlation of HIV infection with *Giardia intestinalis* assemblage B but not with assemblage A in asymptomatic Kenyan children. *AIDS* 30, 2385–2387. [PubMed: 27478987]
28. McHardy IH, Wu M, Shimizu-Cohen R, Couturier MR, Humphries RM (2014) Detection of intestinal protozoa in the clinical laboratory. *J Clin Microbiol* 52, 712–720. [PubMed: 24197877]
29. Mengist HM, Taye B, Tsegaye A (2015) Intestinal parasitosis in relation to CD4⁺ T cells levels and anemia among HAART initiated and HAART naive pediatric HIV patients in a Model ART center in Addis Ababa, Ethiopia. *PLoS One* 10, e0117715. [PubMed: 25658626]

30. Monis PT, Caccio SM, Thompson RC (2009) Variation in *Giardia*: towards a taxonomic revision of the genus. *Trends Parasitol* 25, 93–100. [PubMed: 19135417]
31. Nace EK, Steurer FJ, Eberhard ML (1999) Evaluation of Streck tissue fixative, a nonformalin fixative for preservation of stool samples and subsequent parasitologic examination. *J Clin Microbiol* 37, 4113–4119. [PubMed: 10565940]
32. Nevez G, Le Gal S, Quinio D, Ianotto JC, Berthou C, Hamane S, Sarfati C, Menotti J (2015) *Encephalitozoon hellem* in a patient with CD4⁺ T-cell prolymphocytic leukemia: case report and genomic identification. *Diagn Microbiol Infect Dis* 83, 245–247. [PubMed: 26299583]
33. Ng CT, Gilchrist CA, Lane A, Roy S, Haque R, Houpt ER (2005) Multiplex real-time PCR assay using Scorpion probes and DNA capture for genotype-specific detection of *Giardia lamblia* on fecal samples. *J Clin Microbiol* 43, 1256–1260. [PubMed: 15750093]
34. Oberhuber G, Mesteri I, Kopf W, Müller H (2016) Demonstration of trophozoites of *G. lamblia* in ileal mucosal biopsy specimens may reveal giardiasis in patients with significantly inflamed parasite-free duodenal mucosa. *Am J Surg Pathol* 40, 1280–1285. [PubMed: 27186850]
35. Perlmutter DH, Leichtner AM, Goldman H, Winter HS (1985) Chronic diarrhea associated with hypogammaglobulinemia and enteropathy in infants and children. *Dig Dis Sci* 30, 1149–1155. [PubMed: 4064865]
36. Read CM, Monis PT, Thompson RC (2004) Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infect Genet Evol* 4, 125–130. [PubMed: 15157630]
37. Ryan U, Paparini A, Oskam C (2017) New technologies for detection of enteric parasites. *Trends Parasitol* 33, 532–546. [PubMed: 28385423]
38. Sanyaolu AO, Oyibo WA, Fagbenro-Beyioku AF, Gbadegeshin AH, Iriemenam NC (2011) Comparative study of entero-parasitic infections among HIV sero-positive and seronegative patients in Lagos, Nigeria. *Acta Trop* 120, 268–272. [PubMed: 21896269]
39. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM (2011) Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis* 17, 7–15. [PubMed: 21192848]
40. Schuurman T, Lankamp P, van Belkum A, Kooistra-Smid M, van Zwet A (2007) Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of *Giardia lamblia* in human stool specimens. *Clin Microbiol Infect* 13, 1186–1191. [PubMed: 17949440]
41. Silva RK, Pacheco FT, Martins AS, Menezes JF, Costa-Ribeiro H, Jr, Ribeiro TC, Mattos ÂP, Oliveira RR, Soares NM, Teixeira MC (2016) Performance of microscopy and ELISA for diagnosing *Giardia duodenalis* infection in different pediatric groups. *Parasitol Int* 65, 635–640. [PubMed: 27586394]
42. Solaymani-Mohammadi S, Singer SM (2010) *Giardia duodenalis*: the double-edged sword of immune responses in giardiasis. *Exp Parasitol* 126, 292–297. [PubMed: 20599999]
43. Solaymani-Mohammadi S, Singer SM (2011) Host immunity and pathogen strain contribute to intestinal disaccharidase impairment following gut infection. *J Immunol* 187, 3769–3775. [PubMed: 21873528]
44. Solaymani-Mohammadi S, Singer SM (2013) Regulation of intestinal epithelial cell cytoskeletal remodeling by cellular immunity following gut infection. *Mucosal Immunol* 6, 369–378. [PubMed: 22910215]
45. Strand EA, Robertson LJ, Hanevik K, Alvsvåg JO, Mørch K, Langeland N (2008) Sensitivity of a *Giardia* antigen test in persistent giardiasis following an extensive outbreak. *Clin Microbiol Infect* 14, 1069–1071. [PubMed: 18808422]
46. Thornton SA, West AH, DuPont HL, Pickering LK (1983) Comparison of methods for identification of *Giardia lamblia*. *Am J Clin Pathol* 80, 858–860. [PubMed: 6637891]
47. van Lieshout L, Roestenberg M (2015) Clinical consequences of new diagnostic tools for intestinal parasites. *Clin Microbiol Infect* 21, 520–528. [PubMed: 25843505]
48. Veenemans J, Mank T, Ottenhof M, Baidjoe A, Mbugi EV, Demir AY, Wielders JP, Savelkoul HF, Verhoef H (2011) Protection against diarrhea associated with *Giardia intestinalis* is lost with multi-nutrient supplementation: a study in Tanzanian children. *PLoS Negl Trop Dis* 5, e1158. [PubMed: 21666789]

49. Wolfe MS. Giardiasis. (1992) Clin Microbiol Rev 5, 93–100. [PubMed: 1735095]
50. Yoder JS, Gargano JW, Wallace RM, Beach MJ (2012) Giardiasis surveillance--United States, 2009–2010. MMWR Surveill Summ 61, 13–23. [PubMed: 22951494]
51. Zia-Ali N, Massoud J (1997) A survey on the prevalence of intestinal parasites in Kerman. Journal of the Kerman University of Medical Sciences 3, 129–134.
52. Zijenah LS, Kadzirange G, Madzime S, Borok M, Mudiwa C, Tobaiwa O, Mucheche M, Rusakaniko S, Katzenstein DA (2006) Affordable flow cytometry for enumeration of absolute CD4⁺ T-lymphocytes to identify subtype C HIV-1 infected adults requiring antiretroviral therapy (ART) and monitoring response to ART in a resource-limited setting. J Transl Med 4, 33. [PubMed: 16907973]

Highlights:

- 1-** Stool antigen-based diagnostic modalities are as sensitive as PCR-based techniques for the diagnosis of *G. duodenalis* infection in patients with HIV and cancer
- 2-** AI genotype of *Giardia duodenalis* was the most common parasite found in patients with HIV and cancer
- 3-** Molecular identification of *G. duodenalis* using the *gdh* and *tpi* loci were consistent in patients with HIV and cancer

FIGURE 1.

CD4⁺ T cell absolute counts in HIV-positive human subjects with or without concomitant enteric parasitic infections. Peripheral CD4⁺ T cell counts were determined using a Partec CD4 Easy Count kit. * $p < 0.05$ (a two-tailed Mann-Whitney U test). Scatter plots represent Mean $\pm$ SEM.

FIGURE 2.

Gel electrophoresis of the semi-nested PCR amplification of a 432-bp fragment of the *gdh* locus of *G. duodenalis*. M, DNA ladder (Thermo Scientific); lane 1, positive control; lanes 2–5 patient's clinical samples; lane 6, negative control (no DNA) (A). The restriction enzyme digestion of the PCR product targeting the *G. duodenalis gdh* locus using *BspLI* (*NlaIV*). Lanes 1 and 2, AI genotype; lane 3, AI+B; lane 4, undigested PCR product; M, DNA ladder (B). Gel electrophoresis of the semi-nested PCR amplification of a 476-bp fragment of the *tpi* gene of *G. duodenalis* (C). M, DNA ladder; lane 1, no DNA; lanes 2, 3 undigested PCR products (assemblage A); lanes 4, 5 AII genotypes; lanes 6, 7 AI genotypes. The characteristic band of 15-bp is not visible. Sizes in base pairs are represented by the numbers on the left.

Table 1:

Study group demographics

	Median	Age (y)	Range	Sex	
		Mean $\pm$ SEM		Female (%)	Male (%)
Controls ($n=68$)	31.5	33.31 $\pm$ 2.08	7–72	36 (59)	32 (41)
HIV/AIDS ($n=72$)	40	39.99 $\pm$ 1.12	11–66	26 (36)	46 (64)
Cancer ($n=59$)	45	39.32 $\pm$ 2.67	9 (mon)-74 (y)	21 (35.6)	38 (64.4)

Table 2:Distribution of *G. duodenalis* assemblages/genotypes in human subjects studied

	Age(y)	Sex	Status	Genotype	
				<i>gdh</i>	<i>tpi</i>
Patient 1	26	M	Immunocompetent	AII	AII
Patient 2	45	F	Immunocompetent	AI	AI
Patient 3	37	F	Immunocompetent	AI	AI
Patient 4	13	M	Cancer	AII	AII
Patient 5	25	M	Cancer	AI	AII
Patient 6	53	F	Cancer	AI	AI
Patient 7	6	F	Cancer	AI	AI
Patient 8	38	M	HIV	AII	AII
Patient 9	11	F	HIV	AI+B	A+B
Patient 10	43	M	HIV	AI+B	A+B
Patient 11	41	F	HIV	AI	AI