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Performance of microscopy and ELISA for diagnosing *Giardia duodenalis* infection in different pediatric groups

Renata K.N.R. Silva [a], Flávia T.F. Pacheco [a], Adson S. Martins [a], Joelma F. Menezes [a], Hugo Costa-Ribeiro Jr. [b], Tereza C.M. Ribeiro [b], Ângela P. Mattos [b], Ricardo R. Oliveira [c], Neci M. Soares [a], Márcia C.A. Teixeira [a,*]

[a] Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Brazil
[b] Centro Pediátrico Professor Hosannah de Oliveira, Universidade Federal da Bahia, Salvador, Brazil
[c] Centro de Pesquisas Professor Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil

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ABSTRACT

Techniques for *Giardia* diagnosis based on microscopy are usually applied as routine laboratory testing; however, they typically exhibit low sensitivity. This study aimed to evaluate *Giardia duodenalis* and other intestinal parasitic infections in different pediatric groups, with an emphasis on the comparison of *Giardia* diagnostic techniques. Feces from 824 children from different groups (diarrheic, malnourished, with cancer and from day care) were examined by microscopy and ELISA for *Giardia*, *Cryptosporidium* sp. and *Entamoeba histolytica* coproantigen detection. *Giardia*-positive samples from day-care children, identified by either microscopy or ELISA, were further tested by PCR targeting of the *β-giardin* and *Gdh* genes. Statistically significant differences ($P < 0.05$) were observed when comparing the frequency of each protozoan among the groups. *Giardia duodenalis* was more frequent in day-care children and *Cryptosporidium* sp. in diarrheic and malnourished groups; infections by *Entamoeba histolytica* were found only in children with diarrhea. Considering positivity for *Giardia* by at least one method, ELISA was found to be more sensitive than microscopy (97% versus 55%). To examine discrepancies among the diagnostic methods, 71 *Giardia*-positive stool samples from day-care children were tested by PCR; of these, DNA was amplified from 51 samples (77.4%). Concordance of positivity between microscopy and ELISA was found for 48 samples, with 43 confirmed by PCR. Parasite DNA was amplified from eleven of the 20 *Giardia* samples (55%) identified only by ELISA. This study shows the higher sensitivity of ELISA over microscopy for *Giardia* diagnosis when a single sample is analyzed and emphasizes the need for methods based on coproantigen detection to identify this parasite in diarrheic fecal samples.

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1. Introduction

Children are an important risk group for enteroparasitic diseases, both because their immune system is not fully developed at the first contact with the parasite or because of the habits of infants, such as bringing any object to the mouth or having increased contact with the ground [1]. Moreover, regardless of the integrity of the immune response, chronic infections with intestinal parasites in children can lead to malnutrition, anemia, and growth delay [2].

Giardiasis has a global distribution, and *Giardia* is one of the most common parasites associated with diarrhea in humans. Due to the high prevalence of giardiasis in young children in developing countries and its effects on early childhood diarrhea and malnutrition, giardiasis is of considerable public-health importance [1–3]. In 2004, *Giardia duodenalis*, along with *Cryptosporidium* sp., was included in the "Neglected Diseases Initiative" group of the World Health Organization [4]. The transmission of giardiasis occurs via a fecal-oral route, with infection resulting from the ingestion of cysts present in food or water contaminated with feces [5]. Direct transmission from person to person also contributes to the dissemination of the parasite among children attending day-care centers and schools [1,5,6].

Giardia trophozoites are identified through direct examination of diarrheal stools, whereas the detection of cysts is optimized by concentration methods, such as centrifugal flotation [7] or sedimentation by centrifugation [8]. After concentration, fecal smears can be stained using iodine or iron hematoxylin [9], and microscopy has certain advantages, such as the possibility of simultaneous detection of several parasites, low cost, and ease of implementation [10]. However, due to the intermittency of cyst excretion in feces, the examination of multiple samples is necessary to increase the efficiency of parasitological diagnosis [11,12].

Immunoassays for *Giardia* antigen detection have been used as alternative methods for the diagnosis of giardiasis, and these methods

* Corresponding author at: Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Bahia 40170-115, Brazil.
E-mail address: marciat@ufba.br (M.C.A. Teixeira).

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present high sensitivity and specificity [13,14]. However, the routine use of these kits in the laboratory is controversial due to the high cost in relation to stool examination by microscopy.

Molecular techniques based on the amplification of parasite DNA have emerged and include the polymerase chain reaction (PCR), which has been shown to be a highly sensitive and specific method that allows the detection of *Giardia* DNA directly from fecal samples [15–17]. Nevertheless, a negative result does not rule out the presence of the parasite because interference from PCR inhibitors present in feces may hamper DNA amplification.

The objective of this study was to determine the frequency of *Giardia duodenalis* infection and other intestinal parasites in different pediatric groups with and without health issues and to compare the performance of ELISA and microscopy for *G. duodenalis* diagnosis.

2. Materials and methods

2.1. Samples

Stool samples were obtained from 824 children, divided into the following groups: cancer (n = 70); malnourished (n = 110); diarrheal disease (n = 151); and attending day care centers (n = 493). Sample size for this study was determined using Epi Info software. As the main goal was to compare techniques for *Giardia* diagnosis in different groups, it was taking into account previous studies of giardiasis in pediatric population with similar characteristics. Therefore, the expected prevalence of *Giardia duodenalis* infections were considered to be approximately 6.0% for cancer [18], 10.0% for malnourished [19] 4.7% for diarrheic [20] and 22.1% for day-care children [21–23]. Considering a 90% confidence interval, the minimal sample size determined for the groups studied were 62, 98, 49 and 187, respectively. Thereafter, all children whose parents accepted to participate of the study and signed an informed consent were enrolled during the research period.

All children fitted to general inclusion criteria such: aged under 10 years, users of health public services, from low-income families (one Brazilian minimum salary or less) and whose parents or guardians agreed to participate and signed an informed consent form. Specific inclusion criteria for groups included: a) Cancer – pediatric patients with confirmed neoplastic disease; b) Malnourished – children with diagnosis of severe protein-energy malnutrition, associated to poor diet; c) Diarrheic – patients with acute or persistent diarrhea, with signs of dehydration; d) Day care children – apparently healthy children without any infection/pathology diagnosed. Therefore, for children with healthy issues, the most important inclusion criteria was to have confirmed diagnosis of the base disease by medical practitioners, including pediatricians and/or specialists, such oncologists.

The majority of participants were composed of young children (up to 5 years) in cancer (45/70; 64.3%), malnourished (98/110; 89.1%), diarrhea (133/151; 88.1%) and in day-care (493/493; 100%) groups. Children hospitalized at the Hospital of Federal University of Bahia, Brazil, comprised the malnutrition and diarrheic pediatric groups. Children with cancer were outpatients of the same hospital, assisted by ambulatory or laboratory services. Of the 70 children with neoplastic diseases, 40 (57.1%) had acute lymphocytic leukemia or myelogenous leukemia, whereas 12 (17.1%) had malignant brain tumors and 9 (12.9%) lymphoma. The 9 (12.9%) remaining patients included children with retinoblastoma, abdominal neuroblastoma and thyroid carcinoma. Apparently healthy children without any infection/pathology diagnosed were recruited from two day care centers located in the same city district and supported by a social institution.

The study was conducted from January 2011 to June 2012. One single stool specimen was collected from each child and immediately transported to the Parasitology Laboratory of Pharmacy Faculty of Federal University of Bahia for processing. This study was approved by the Ethics Committee of the Department of Health of Bahia, and all parasitological test results were sent to the children's parents or the respective pediatrician or oncologist. Individuals found positive for pathogenic intestinal parasites were treated with appropriate drugs by their doctors.

2.2. Diagnosis of intestinal parasites in fecal samples

Stool samples from malnourished, cancer and day-care children were mostly formed or soft and were subjected to six parasitological methods: a) direct examination; b) the Baermann-Moraes technique [24]; c) stool culture on agar plate [25]; d) zinc sulfate (density of solution 1.18 g/ml) centrifugal flotation [7]; e) sedimentation by centrifugation in water [8] and f) modified Ziehl-Neelsen staining [26]. Diarrheal stool samples were examined by all methods cited above, except for the Baermann-Moraes method due to well-known technical limitations for liquid stools.

Fecal pellets obtained by the sedimentation technique were tested for helminths and protozoa by wet mounts with saline and iodine, as well as staining with modified Ziehl-Neelsen for *Cryptosporidium* microscopical diagnosis. Two slides were examined for each technique. Besides the parasitological examination, all samples from the four groups of children were tested by ELISA for coproantigen detection of *Giardia duodenalis*, *Cryptosporidium* sp., and *Entamoeba histolytica* (Wampole II *Cryptosporidium*, *Giardia* II, and *E. histolytica* II, TECHLAB, Blacksburg, VA, USA), except for 12 samples from the malnourished and 16 from the diarrheic children due to insufficient sample.

2.3. Comparison between ELISA and microscopy for the diagnosis of Giardia duodenalis in fecal samples

Considering the irregular fecal cyst excretion in asymptomatic hosts and the reduced viability of trophozoites in diarrheal specimens, the use of different diagnostic methods is necessary to increase the sensitivity of parasite identification in fecal samples. In this study, the diagnosis of *G. duodenalis* in fecal samples was performed by microscopy - through direct examination, centrifugal sedimentation and flotation in zinc sulfate - and by ELISA for *Giardia* coproantigen.

For a comparison analysis between *G. duodenalis* diagnosis by ELISA and microscopy, only 796 fecal samples were tested due to a lack of sufficient material for 12 samples from the malnourished children and 16 from the group with diarrhea. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy kappa coefficient were evaluated by two different approaches, as follows: a) evaluation of ELISA considering microscopy as the gold standard and b) evaluation of microscopy and ELISA considering the combined results of all methods tested.

2.4. Assessment of discrepancy between microscopy and ELISA results using PCR for G. duodenalis identification in feces

Seventy-one *G. duodenalis*-positive stool samples from day-care children diagnosed by microscopic and/or ELISA, as described above, were subjected to PCR to evaluate discordant results between the diagnostic methods. Of these, 3 samples were identified only by microscopy, 20 exclusively by ELISA, and 48 by both methods. *Giardia* PCR was conducted only with samples from the day-care children because this group provided most of the positive samples (134/152) as well as sufficient stool for DNA extraction.

DNA from *G. duodenalis* cysts was purified using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with some modifications. For example, the time and temperature of the cell lysis step were increased to 10 min at 95 °C, and the DNA elution volume was reduced to 100 μl of buffer.

A 753-bp fragment of the *β-giardin* gene was amplified using forward primer G7 and reverse primer G759, as described by Cacciò et al. [27]. In the sequential nested PCR reaction, a 511-bp fragment was amplified using forward primer G99 and reverse primer G609, as described

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