

Detection of anti-*Giardia lamblia* serum antibody among children of day care centers

Detecção de anticorpos séricos anti-*Giardia lamblia* em crianças de creches

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Keywords

Giardia lamblia, immunology.[#]
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Descritores

Giardia lamblia, imunologia.[#]
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Abstract

Objetives

To detect anti-*Giardia lamblia* serum antibodies in healthy children attending public day care centers and to assess serological tests as tools for estimating the prevalence of *G. lamblia* in endemic areas.

Methods

Three separate stool specimens and filter paper blood samples were collected from 147 children ranging from 0 to 6 years old. Each stool sample was processed using spontaneous sedimentation and zinc sulfate flotation methods. Blood samples were tested by indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA) for *Giardia* IgG.

Results and Conclusions

Of 147 individuals tested, 93 (63.3%) showed *Giardia* cysts in their feces. Using IIF and ELISA, serum antibodies were detected in 93 (63.3%) and 100 (68%) samples, respectively. Sensitivity of IIF and ELISA was 82% and 72%, respectively. However, ELISA revealed to be less specific (39%) than IIF (70%). IIF also showed a higher concordance with microscopic examination than ELISA.

Resumo

Objetivos

Detectar anticorpos séricos anti-*Giardia lamblia* entre crianças atendidas em creches e estimar a frequência de infecção por *Giardia lamblia* em área endêmica.

Métodos

Foram coletadas três amostras de fezes de cada uma das 147 crianças de três creches da rede municipal de Botucatu, SP, com idade variando de 0 a 6 anos, e as amostras foram processadas pelos métodos de sedimentação espontânea e flutuação pelo sulfato de zinco. Amostras de sangue foram obtidas da polpa digital, coletadas em papel de filtro e testadas pelos métodos de imunofluorescência indireta (IFI) e de reação imunoenzimática (Elisa) para pesquisa de IgG anti-*Giardia*.

Resultados e Conclusões

De um total de 147 crianças, 93 (63,3%) apresentaram cistos de *Giardia* nas fezes. Dos 147 eluatos testados, 93 (63,3%) e 100 (68%) foram positivos para *Giardia* em IFI e em Elisa, respectivamente. A sensibilidade de IFI foi de 82% e de Elisa, 72%. Contudo, Elisa foi menos específica (39%) do que IFI (70%). A imunofluorescência indireta apresentou maior concordância com o exame de fezes do que Elisa.

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INTRODUCTION

Giardia lamblia is a common human intestinal parasite of worldwide distribution found in both developed and developing countries. In endemic areas, high prevalence rates for *G. lamblia* infection have been reported in children, mainly among nursery and primary school children.^{2,5,6,9,13}

Giardia infection is usually diagnosed under light microscopy to identify either trophozoites or cysts in feces samples. In general, the examination of *Giardia* is performed in a single stool sample. However, as this parasite presents a variable pattern of excretion, misdiagnoses have been common and the actual prevalence may be underestimated. Microscopy examination of duodenal aspirate and jejunal biopsies is sometimes necessary. Since these two methods are invasive, they are rarely employed in children.

Given that infected individuals have anti-parasite antibodies, in an effort to improve the tools for *Giardia* diagnosis, different methods have been developed for detection of anti-*Giardia* antibodies in serum, particularly specific IgG.^{1,15,16,18-20} Most of these studies however were carried out among symptomatic giardiasis cases, but a large proportion of infected individuals are asymptomatic.

The present study was performed to compare serum anti-*Giardia* IgG antibody levels detected using indirect immunofluorescence (IIF) and ELISA to the frequency of infection in three stool examinations among children of day care centers in an endemic area. Also, the purpose was to verify whether serodiagnosis could be used to determine infection frequency in certain population groups, mainly asymptomatic cases. It is important to emphasize that immunologic assays were conducted using antigens prepared from trophozoites of an axenic strain isolated in Brazil and blood samples were collected on filter paper.

METHODS

Stool and blood samples

The studied population consisted of 147 children ranging from 0 to 6 years old who attended three public day care centers in the city of Botucatu, state of São Paulo, Brazil.* Blood and feces samples were collected from children only after obtaining a formal consent signed by their parents.

Three stool specimens collected with a 7-day interval were examined using zinc sulfate flotation³ and

spontaneous sedimentation¹⁴ methods. Blood samples obtained by finger tip lancet pricking were absorbed on Whatman filter paper #1, air-dried at room temperature and stored at -20°C until tested. A calibrated punch was applied to ensure discs measuring 1.32 cm^2 which were incubated in 0.4 ml phosphate buffered saline solution (PBS) at pH 7.2 for 30 min. at room temperature, corresponding to a 1:20 serum dilution according to Souza & Camargo¹⁷ (1966). Positive and negative controls were run in all assays.

Antigen preparations

Trophozoites of BTU-1 strain were employed to prepare antigens for IIF, ELISA, and absorption techniques. This strain was obtained from cysts found in the feces of a 7-year-old asymptomatic child and processed at the university's giardiasis laboratory (IB/UNESP). Trophozoites were grown axenically in TYI-S-33 medium supplemented with bile¹² at 37°C .

Indirect immunofluorescence (IIF) assay

An equal volume of 1% formalin was added to trophozoites before overnight incubation at room temperature. The solution was washed three times with PBS, pH 7.2 and a concentration of about 40 organisms per 40x microscope field was used as antigen on the slide. Slides let to dry out at room temperature and stored at -70°C until used.

IIF assay was performed according to Vivesvara et al²¹ with modifications. Eluates were used in a 1:20 serum dilution. One drop of each sample per well was added to antigen slides for 30 min. at 37°C . The slides were washed with PBS, pH 7.2 and goat anti-human IgG fluorescein isothiocyanate conjugates were added to each well at a 1:40 serum dilution. After 30 minutes, the slides were washed out and examined after mounting in glycerol buffered saline. A titer equals to or greater than 1:40 was considered positive.

ELISA assay

It was performed a modified assay according to Miotti et al¹⁵ (1985). Trophozoites were centrifuged at 250 xg for 10 min. at 4°C , and the supernatant was discharged. The mass culture was re-suspended in PBS, pH 7.2 and washed twice as described above. The washed organisms were adjusted to a concentration of 10^4 trophozoites/50 μl , and then stored at -70°C until used.

Fifty microliters (50 μl) of trophozoite suspension (10^4) per well were used to coat plates. The plates

*This study was approved by the Ethics Committee of the "Faculdade de Medicina da Universidade Estadual Paulista" and of the "Secretaria Municipal de Educação"

were incubated overnight at 4°C, washed 3 times in PBS, pH 7.2 containing 0.05% Tween 20 (PBST), and then blocked for 1 hour with 150 µl/well of PBS with 5% skim milk. After 3 washes with PBST, 50 µl of each blood spot eluate 1:40 diluted were added to a well and incubated for 1 hour at room temperature. After washing, 50 µl of anti-IgG peroxidase conjugated diluted 1:4000 in PBS – 1% skim milk were added to each well for 1 hour at room temperature. The enzymatic reaction was developed with 2 mg of o-phenylenediamine in 10 ml of phosphate citrate buffer, pH 5.0 upon addition of hydrogen peroxide. The reaction was interrupted by the addition of 25 µl of 4N sulfuric acid and the intensity of color was recorded as optical density (OD) at 492 nm wavelength. The cut-off value was calculated as the arithmetic mean of the negative control samples plus two standard deviations ($X \pm 2SD$).

Absorption technique

Antigens of *Ascaris lumbricoides* and *Hymenolepis nana* were prepared for absorption technique. A crude saline extract of homogenized worm was employed for obtaining *A. lumbricoides* antigens. *H. nana* antigen was obtained from a crude saline extract of worms collected from egg-inoculated mice.

The absorption technique was processed according to Wittner et al (1983).²⁰ One-half milliliters of positive-titer eluate was incubated with 0.5 ml of sedimented antigen for 2 hours at 37°C and overnight at 4°C. After centrifugation at 600 xg for 20 minutes, the absorbed eluate was ready to use in both IIF and ELISA assays.

A positive eluate for *Giardia* was used for absorption with *Giardia lamblia* (25 µg of protein), *Ascaris lumbricoides* (25 µg of protein) and *Hymenolepis nana* (30 µg of protein).

Data analysis

The correlation between microscopic examination of stool samples and serologic methods (IFI and ELISA) was analyzed using the McNemar test.⁴ The concordance between each serologic method (IIF and

ELISA) and stool examination was determined considering both positive and negative cases for each method.

ELISA and IFI titers proportions according to stool examination results were analyzed using the Goodman test.^{7,8}

IFI and ELISA sensitivity and specificity were determined regarding the microscopic visual interpretation of slides and OD values, respectively, where:

$$\text{Sensitivity \%} = \frac{\text{Total samples both positive on microscopic examination and serologic test}}{\text{Total samples positive on microscopic examination}} \times 100$$

$$\text{Specificity \%} = \frac{\text{Total samples both negative on microscopic examination and serologic test}}{\text{Total samples negative on microscopic examination}} \times 100$$

RESULTS

Microscopic examination of stool samples obtained from 147 children revealed that 93 (63.3%) were infected with *G. lamblia*. Only *Giardia* cysts were identified in all positive samples. No trophozoites were found in the stool samples. The highest percentages of infection were observed in children aged 12 to 47 months, and within this group the higher percentage of infected (18.3%) was seen in children aged 36 to 47 months. Other intestinal parasites found included *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis*, *Ancylostomatidae*, *Strongyloides stercoralis*, *Hymenolepis nana*, *Entamoeba coli*, and *Blastocystis hominis*. Forty (43%) of 93 stool samples with *Giardia* cysts and 20 (37%) of 54 negative samples had at least another intestinal parasite.

Serum antibodies to *G. lamblia* were detected in 93 (63.3%) and 100 (68%) of the 147 eluates using IIF and ELISA, respectively (Table 1).

In IIF assay, antibody titers were invariably low ranging from 1:40 to 1:80 (Table 2). Of 93 positive samples in IIF, 64 were positive at 1:40 (Table 2).

Table 1 - Comparison of microscopical examination of three stool samples, IIF and ELISA for *Giardia lamblia* infection.

Stool examination	Positive (%)	IIF		Total	Positive (%)	ELISA		Total
		Positive (%)	Negative (%)			Positive (%)	Negative (%)	
Positive	76*(81.7)	17 (18.3)		93	67*(72.0)	26 (28.0)		93
Negative	17 (31.5)	37*(68.5)		54	33 (61.1)	21*(38.9)		54
Total	93 (63.3)	54 (36.7)		147	100 (68.0)	47 (32.0)		147

*Values considered for calculation of concordance between each immunologic test and examination of stool samples. The concordance was determined with 95 per cent confidence interval.

The analysis of IIF titer proportions according to stool examination revealed that the proportion of infected and seropositive children was higher at 1:40 dilution. For negative cases, the titers were lower than 1:40.

ELISA titers were also low and ranged from 1:40 to 1:640 (Table 3).

Most of the eluates obtained from children with positive stools were tightly bunched. There was an overlapping of these values with those of children without cysts in feces. The highest titers were observed among eluates obtained from children with cysts in feces. Some positive samples showed titers lower than the negative ones (Table 3). As for the analysis of ELISA titer proportions, there were no significant differences among <1:40, 1:40, 1:80 and 1:160 titers. In negative cases, no significant difference was observed between <1:40 and 1:40 titers, but there was a difference when they were compared to 1:80, 1:160, 1:320 and 1:640 titers.

Using IIF, of the 93 eluates of children with cysts in the feces and 54 of those with negative stools, antibodies were detected in 76 (81.7%) and 17 (31.5%), respectively (Table 1). Alternatively, using ELISA, among positive and negative microscopically diagnosed cases, antibodies were detected in 67 (72%) and 33 (61.1%), respectively (Table 1). Thus, IIF sensitivity and specificity was 82 % and 70 % respectively, whereas ELISA sensitivity and specificity was 72% and 39%, respectively.

Table 2 - Indirect immunofluorescence titers for anti-*Giardia* IgG performed on filter paper blood samples from day-care children according to microscopical examination of three stool samples.

Stool examination	Titers			Total
	<1:40 N. (%)	1:40 N. (%)	1:80 N. (%)	
Positive	17 (18.3)	54 (58.1)	22 (23.6)	93
Negative	37 (68.5)	10 (18.5)	07 (11.1)	54
Total	54 (36.7)	64 (43.5)	29 (19.7)	148

Note: The eluates were considered nonreactive when the titer was lower than 1:40.

Table 3 - ELISA titers for anti-*Giardia* IgG performed on filter paper blood samples from day-care children according to microscopical examination of three stool samples.

Stool examination	Titers						Total
	<1:40 N. (%)	1:40 N. (%)	1:80 N. (%)	1:160 N. (%)	1:320 N. (%)	1:640 N. (%)	
Positive	26 (27.9)	27 (29.0)	19 (20.4)	18 (19.3)	02 (2.1)	01 (1.0)	93
Negative	21 (38.8)	26 (48.1)	05 (9.2)	02 (3.7)	—	—	54
Total	47 (31.9)	53 (36.1)	24 (16.3)	20 (13.6)	02 (1.36)	01 (0.68)	147

Note: The eluates were considered nonreactive if the OD < mean OD + 2.0 SD of controls at a 1:40 dilution.

The concordance between IIF assay and the 7-day-interval examination of three stool samples was 76.9%. When ELISA was compared to microscopy examination the concordance was 59.9%.

All absorption tests performed with *Giardia* trophozoites against a positive eluate revealed a significant reduction in titers from the pre-absorption values. No variation was seen when the same eluate was absorbed against *A. lumbricoides* and *H. nana* antigens.

DISCUSSION

Though high prevalence rates of *G. lamblia* infection have been found in children of endemic areas, there are few serologic studies in these groups. In view of that *Giardia* is endemic in Brazil, and there are no seroepidemiological studies in children, the purpose of this study was to compare IIF and ELISA IgG of filter paper blood samples to three stool examinations with a 7-day interval between collection.

Most serologic studies have been able to detect *Giardia* infection among symptomatic individuals. In general, the anti-parasite antibody response of symptomatic cases is stronger than that of asymptomatic individuals, and is related to the magnitude of cyst output.¹⁶ However, it is noteworthy that in endemic areas most infected individuals are asymptomatic carriers.

In the present study, when only asymptomatic individuals were enrolled, IIF showed a higher concordance than ELISA. IIF was more sensitive and showed higher specificity than ELISA. By its turn ELISA showed a high percentage of false-positive among samples of children without cysts in feces. Wittner et al²⁰ compared the same immunologic methods employing serum of symptomatic and asymptomatic individuals. It was observed that ELISA was also less specific than IIF, yielding more false-positive results among the groups without *Giardia* (58%). These authors raised the question whether this result could represent a measure of cross-reactivity or previous exposure to *Giardia*.

According to Soliman et al¹⁶ (1998), in areas of high endemicity, the continuous, high level exposure of humans to the parasite early in life induces a stronger circulating anti-parasite antibody response that transects different age groups to the population.

Consistent with previous studies in endemic areas,^{6,10} it was not possible to establish a correlation between infection and the presence of circulating antibodies. Besides, in cases with and without *Giardia* cysts in the feces, an overlap of titers was observed when both serologic methods were used to test the eluates.

Storing blood samples stored as dried spots on filter paper was tested by Al-Tukhi et al¹ (1993) and it was showed that this method is applicable to seroepidemiological studies in giardiasis. In this study, the main advantage of this method was to ease blood samples collection from 0 to 6-year old children, and facilitate their transport and storage.

As for the study results, IIF and ELISA did not show a satisfactory concordance with stool examination, but the prevalence of seropositivity was high

for both methods, 62.8 % and 68.2 %, respectively, in comparison to stool examination. Gilman et al⁶ (1985) in a study conducted in Bangladesh, a highly endemic *Giardia* area, observed a poor concordance between stool examination and serum antibody titers, showing that in areas with high infection rates, a single serum antibody titer cannot be used as a marker for *Giardia* infection. Thus it was suggested that antibody titers may be an useful epidemiological tool to determine the frequency of infection in endemic focus. According to Jokipii et al¹¹ (1988), despite exhaustive serologic studies, the results have been discrepant, varying from distinctive to overlapping titers in individuals with and without *Giardia* infection. It is important to note that not all discrepant results should be rejected since they can be due to: (1) the use of different strains of *Giardia* in antigen preparation; (2) enrolling only symptomatic patients in most of the studies; (3) choice of different serologic techniques and their various modifications.

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