Original Article

Giardia Diagnostic Methods in Human Fecal Samples: A Comparative Study

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Several methods were tried for *Giardia* detection in stool. This study aimed to compare between the results of ordinary microscopy, direct immunofluorescence assay (DIF), and flow cytometry (FC) for the detection of *Giardia* cyst in human stool samples. The study included 84 children recruited from outpatient clinics of Mansoura University Children Hospital. Fecal samples were processed and examined for *Giardia* cysts using conventional microscopy, DIF, and FC. Among 84 fecal samples, 40 (47.6%) were diagnosed as *Giardia*-positive by saline wet mount, while DIF and FC detected 52 (61.9%), and 38 (45%) *Giardia*-positive cases, respectively. When compared with DIF as a gold standard method, ordinary microscopy had 76.9% sensitivity and 100% specificity while the FC had a sensitivity of 73.1% and 100% specificity, with statistically significant differences between DIF and the other two methods ($P < 0.05$). DIF was able to detect as few as 500 cysts/g of concentrated stool, yielding a threshold higher than ordinary microscopy (1,800 cyst/g) even after concentration. It is concluded that direct microscopic examination is reliable in *Giardia* diagnosis as a first choice test. DIF is an excellent technique in clinically suspected cases after negative microscopy. FC was found to be less sensitive to obtain accurate organisms' count but it could be an effective alternative method for the detection of *Giardia* cysts, especially for large-scale epidemiological studies or extensive surveillance programs as it has the beneficial attribute of speed and do not depend on an experienced microscope viewer. However, DIF remains the gold standard while FC still requires significant technical improvements before it can compete with DIF for *Giardia* diagnosis. © 2012 International Clinical Cytometry Society

Key terms: *Giardia* diagnosis; direct immunofluorescent antibody; flow cytometry

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Giardia nowadays is recognized as the most common parasitological cause of diarrhea, with 280 million infections per year. Giardiasis is a frequently diagnosed waterborne infection and a major concern to drinking water authorities. Because of the impact on socioeconomic development, as well as on domestic animals such as cattle and sheep especially in developing countries, *Giardia* is included in the "Neglected Disease Initiative" of the World Health Organization (1,2).

Clinical parasitology laboratories, in contrast to most other diagnostic laboratories, utilize many complex manual technical procedures that are subjected to individual variation and subjective interpretations (3). Sensitivity is poor when only a single sample is analyzed, particularly if there is low parasite density, insufficient microscopic quality, intermittent excretion of cysts or the probability of parasite hiding by bile pigments (4). Microscopic examination must be performed on three stool samples to increase sensitivity (5). This leads to problems concerning patient compliance and delays the final diagnosis (6). The sensitivity of laboratory diagnosis of *Giardia lamblia* infection can be improved by including alternative diagnostic procedures which are more rapid and reliable (7,8).

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Direct immunofluorescent antibody (DIF) test, based on binding of specific fluorescent monoclonal antibodies to *G. lamblia* cysts, was used for diagnosis with high sensitivity and specificity (9). In addition, FC has been suggested as an effective method, with significant advantages. An optimized FC protocol for *G. lamblia* provides an accurate, fast, simple and automated detection method for clinical diagnosis and water analysis (10). In FC, the identification of *Giardia* cysts is facilitated due to improved staining of cysts caused by exposure of more epitopes to the monoclonal antibody in suspension than when fixed to a glass slide. Besides, larger volume of samples can be analyzed, providing more accurate description of the samples compared to fluorescence microscopic examination (11).

The aim of this study is to compare conventional microscopy, DIF, and FC as diagnostic methods for *Giardia* diagnosis and to assess the efficiency of FC in such diagnosis.

MATERIAL AND METHODS

This study was conducted in the period from May 2009 to August 2010. Eighty-four children from outpatient clinics of Mansoura University Children Hospital were enrolled in this study: 46 males and 38 females, the mean age of examinees was 6.8 ± 2.01 (range: 2–11 years old). The purpose of the study was explained, and verbal consents were obtained from parents of all participants.

Fecal Sample Collection and Preparation

Fecal samples were collected in clean wide mouth containers and examined immediately with wet mount smear and formol–ether sedimentation methods. One gram from each sample was then transferred into each of two tubes containing 5 ml of 10% buffered formalin as a preservative to prepare suspensions. These tubes were labeled according to ID number and date of collection, and kept refrigerated at 4–8°C until subsequent immunological analysis.

Conventional Microscopy

One drop of fecal suspension was transferred to a microscope slide with a cover slip. Each slide was then examined as a direct mount at 40× magnification and presence or absence of *G. lamblia* cysts was recorded. For further confirmation, formol–ether concentration technique was performed.

Immunological Methods

Stool samples preserved in 10% buffered formalin and kept refrigerated at 4–8°C were tested by DIF (*Giardia*-Cel, Cellabs, Brookvale, Australia) and FC (*Giardia*-a-Glo, Waterborne, New Orleans, LA).

Direct immunofluorescence assay. Following formol–ether concentration, 20 μl of each fecal suspension was transferred to a microscope slide and air dried. Slides were then fixed for 5 min in acetone and air dried. Twenty-five microliters of fluorescein-labeled anti-*Giardia* monoclonal antibody (Cellabs *Giardia*-Cel reagent) was added to each slide. Slides were incubated in a humidity chamber for 30 min at 37°C, washed by irrigation in phosphate buffered saline (PBS), excess moisture was removed, and a drop of supplied mounting fluid was added along with a cover slip. A positive control slide provided with the kit was processed in the same manner. Slides were examined immediately at 40× magnification using a fluorescence microscope with filter system for fluorescein isothiocyanate (FITC). The presence or absence of *G. lamblia* cysts was recorded for each sample.

Flow cytometry. Samples processing for FC included formol–ether concentration step followed by a simplified processing method (12). The final step involved extensive vortexing to break up the particulate and centrifugation to separate cysts from formalin and excess antibody. Following formol–ether concentration, 500 μl of the aliquot was centrifuged at 3,000 rpm for 10 min; the pellet was resuspended in 10% buffered formalin to a final volume of 1 ml. The sample was vortexed and then centrifuged at 3,000 rpm for 30 min, the pellet containing cysts was then resuspended in 1 ml of PBS and vortexed for 30 sec. From this suspension, duplicate 200 μl of suspension was removed and placed into 5-ml round bottom tubes. Twenty-five microliters of anti-*Giardia* FITC 1× monoclonal antibody solution (*Giardia*-a-Glo, Waterborne, LA) was added to one tube of each sample, and 25 μl of PBS was added to the duplicate tube as an autofluorescence control. Samples were incubated at room temperature for 45 min in the dark, mixed twice during incubation and the mixture was washed with 2 ml PBS then centrifuged at 3,000 rpm for 20 min. The supernatant was discarded and the pellet was resuspended in PBS to a final volume of 500 μl. All samples were analyzed on the same day of processing on COULTER EPICS XL flow cytometer (Beckman Coulter, CA) with acquisition software (XL SYSTEM II). Acquisition settings were defined using a positive *Giardia* control provided with Giardia-a-glo reagent kit (Waterborne, New Orleans, LA). A maximum of 100,000 events were analyzed for each sample. Gate setting was done using the positive control of pure cysts provided with the Giardia-a-Glo reagent kit; a region based on size versus complexity [forward scatter characteristics (FSC)/side scatter characteristics (SSC)] was set to include over 99% of the cysts. As long as the *Giardia* cysts in the purified stool samples were scattered much more and were not homogenous as in the positive control of pure cysts, analysis was conducted using different window sizes and places for gating including no gate at all, but it was found that the predefined gate on the positive control of pure cysts had the highest sensitivity in comparison to DIF. Consequently, only events falling within the regions defined for pure cysts by FSC/SSC plots were counted as cysts in the samples and stored to list mode files. Dual parameter histograms of event count versus fluorescence intensity were plotted for each sample. Every sample's auto-fluorescence control was analyzed in the same manner to ensure that any fluorescent debris did not appear in the analysis gate. A negative stool sample examined by DIF was

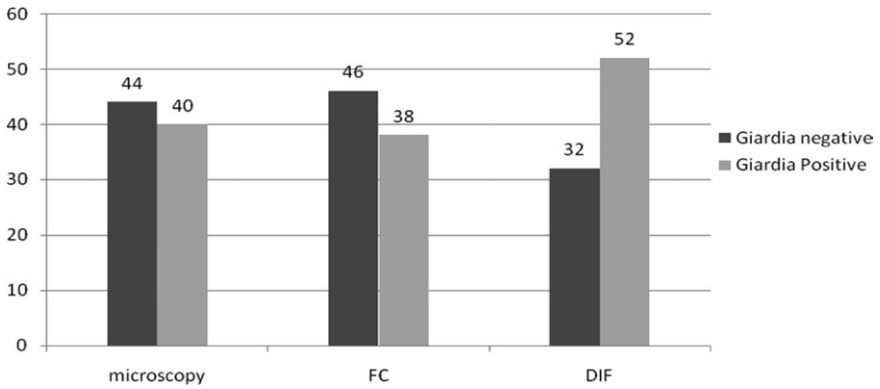

FIG. 1. Giardia results using microscopy, FC, and DIF (84 samples).

considered as the negative control. After each sample run, the system was flushed with deionized water to prevent cross-sample contamination. All comparisons were made against the negative control.

Selection of gold standard test. On the basis of 100% sensitivity and specificity in validation studies in humans (13) as well as its frequent use as a reference standard in numerous studies (14,15), DIF assay was chosen as the reference test in this study.

Cyst count. The number of *Giardia* cysts/g of feces were calculated after formol–ether concentration technique using the formula $N = S/(V \times W)$; while N is the number of cysts/g of feces, S is the number of cysts counted on the slide, V is the volume of sample examined, and W is the stool weight in grams (16).

Statistical Analysis

Data entry and analysis were performed using SPSS (Ver. 17 for Windows) and Medcalc software. The agreement between each test and the gold standard test was determined based on the calculated κ (*kappa*) value gradation (17). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each test were calculated and compared to the gold standard. The results of $P < 0.05$ were considered statistically significant. FC cut-off values were calculated using ROC curve in reference with DIF as a gold standard test. Samples were considered positive if test value was higher than predetermined cut-off.

RESULTS

This study was conducted to evaluate three different methods used for detection of *G. lamblia* in human stool samples (Fig. 1, Table 1). As a gold standard method in our study, DIF revealed 52/84 *Giardia*-positive cases (61.9%) while copromicroscopy diagnosed only 40/84 cases (47.6%) as *Giardia*-positive (38 with wet mount and additional two with formol–ether sedimentation method). Accordingly, ordinary microscopy in this study had a sensitivity of 76.9%, a specificity of 100%, with PPV of 100% for positive result (no false positive result) and NPV of only 72.7% for negative result (Table 2).

As regards FC, its results were evaluated in comparison with DIF results using ROC curve to determine the optimal cut-off point that allows reliable discrimination between positive and negative cases; it was calculated to be 33 events. When the FC results were dichotomized at a cut-off point of 33 events, 38/84 stool samples (45.2%) were classified as *Giardia*-positive using FC with none of these samples considered negative by DIF (no false positive). On the other hand, 46/84 cases (54.8%) were considered negative using FC, 14 of which were positive with DIF (false negative). Accordingly, FC was found to have 73.1% sensitivity and 100 % specificity with positive predictive value of 100% (no false positive result) and a negative predictive value of 69.5%. Examples of FC results are shown in Figure 2.

Among the 44 microscopy negative cases, 12 cases were positive using DIF (microscopy false negative cases). The lowest cyst count detected by microscopy in this study was 1800 cysts per gram stool. While in the 12 microscopy false negative cases, cyst count using DIF was 500–1,500 cysts per gram stool which is lower than that detected by microscopy.

DISCUSSION

Giardiasis is one of the most common pathogenic intestinal protozoan infections worldwide. In this study,

Table 1
Microscopy and FC Versus DIF in Diagnosis of Giardiasis

		DIF			
		Negative $n = 32$ (%)	Positive $n = 52$ (%)	κ	*P*
Microscopy	Negative	32 (100%)	12 (23.1%)	0.748	<0.001
	Positive	0 (0%)	40 (76.9%)		
FC	Negative	32 (100%)	14 (26.9%)	0.713	<0.001
	Positive	0 (0%)	38 (73.1%)		

DIF, diffuse immunofluorescence; FC, flow cytometry.

Table 2
Sensitivity and Specificity of Different Tests Regarding DIF as a Gold Standard

	Microscopy (%)	FC (%)
Sensitivity	76.9	73.1
Specificity	100	100
PPV	100	100
NPV	72.7	69.5
Accuracy	85.7	83.3

FC, flow cytometry; PPV, positive predictive value; NPV; negative predictive value.

we compared three diagnostic methods for the detection of *Giardia* cysts in human feces, namely, copromicroscopy, DIF, and FC. Eighty-four stool samples were examined using the three methods to elucidate their sensitivity, specificity, and applicability.

Although DIF requires the more costly fluorescent microscope but the high sensitivity and specificity makes it ideal for confirming the diagnosis when the infection is suspected clinically but the causative agent cannot be demonstrated (8). Traub et al. (18) concluded that the DIF has a high PPV (90.7%) when used to test human samples, which indicates that this test can be used to diagnose or exclude *Giardia* infection in normal members of the community instead of the three consecutive stool samples required to improve the diagnostic sensitivity of microscopic examination. DIF also reported 100% sensitivity and specificity in validation studies in human (12) and is frequently used as a

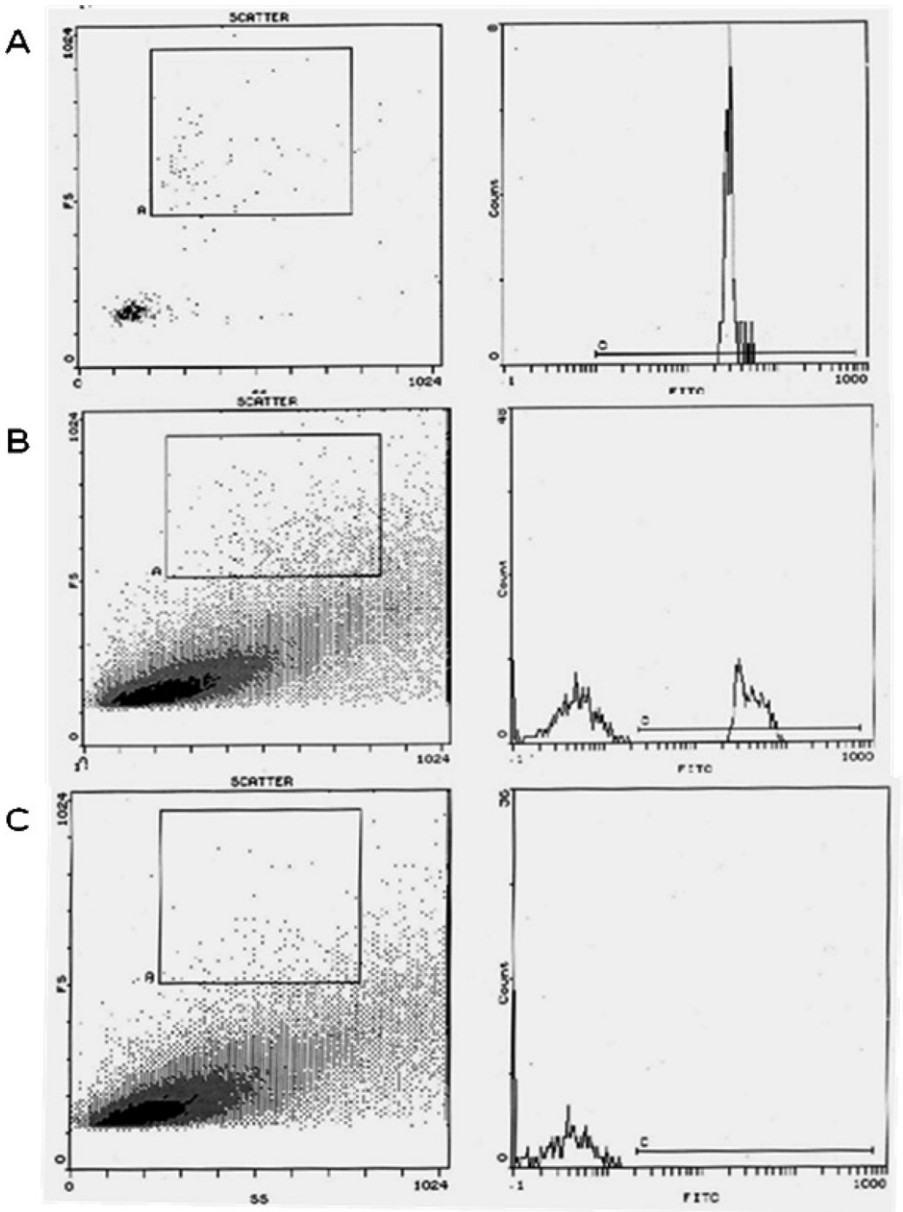

FIG. 2. *Giardia* cyst detection using flow cytometry. (A) *Giardia* positive control, (B) *Giardia* positive sample, (C) *Giardia* negative sample.

reference standard in several studies (13,14). Based on this, DIF is selected as the gold standard test in our study.

Comparing copromicroscopy with DIF as a gold standard test in this study, it demonstrated 76.9 % sensitivity, 100% specificity, with PPV of 100% (no false positive result) and NPV of 72.7% (Table 2). Several other studies reported that the sensitivity of a single stool examination in giardiasis varied from 74 to 89% (19–21), which is in concordance with our study. The lower sensitivity of copromicroscopy is multifactorial and could be explained by the intermittency and paucity of excretion of the diagnostic stages in the stool, the improper methods of specimen collection and handling, and obscuring the parasites by medications and anti-diarrheal drugs (22).

As the number of *Giardia* cysts on the DIF slides decreased, false negatives by copromicroscopy became more common (23). In our study, a threshold level down to 500 cysts/g stool was identified by DIF; this threshold was considered false negative by ordinary microscopy as the lowest cyst count detected by microscopy was 1800 cysts/g stool. These detection limits depend on the concentration techniques used and the characteristics of the stool specimens as the detection limit from formed stool specimens is relatively high due to poor cyst recovery and interference from fecal materials (24–26).

The results of FC in our study showed 73.1% sensitivity and 100 % specificity with 100% PPV (no false positive result) and 69.5% NPV. Conflicting results were found regarding the application of FC in diagnosis of fecal parasites. Some previous studies showed that FC can be successfully applied in the diagnosis of *Giardia*, *Cyclospora*, and *Cryptosporidium* shed in feces (27,28). Dixon et al. (29) compared microscopy, DIF, and FC in the detection of *Giardia lamblia* cysts and proved that fluorescently labeled *Giardia* cysts were readily detected. Another study reported that FC had the same specificity as conventional staining methods in detection of *Cyclospora* in human stool samples, while it was inferior to RT-PCR (30). Gruden et al. (31) stated that despite the speed, sensitivity, and reproducibility, documented FC applications in complex environmental matrices are virtually nonexistent as these samples pose the challenge of a broad range of biogeochemical conditions (ionic strength, pH, particulate matter), which may impact label specificity, fluorescent response, and method sensitivity. Uehlinger et al. (25) reported that the subjectivity in the counting process by FC introduced by the operator's decision ,on what to include in or exclude from the counting process with electronic gating, makes FC inferior to DIF for *Giardia* detection in stool.

The lower sensitivity of FC could be multifactorial resulting from the presence or absence of background fluorescence caused by phototrophic pigments and some organic compounds, the heterogeneity of the sample, the fluorescence distribution, the signal-to noise ratio, the adequate cell recovery, and the efficient hybridization between the probe and the target biological molecule (32). Also, samples with inorganic turbidity (e.g. soil or sediment extracts) often require special pretreatment before analysis (33). Another probable cause that decreased the potential sensitivity of FC is the dilution of the samples prior to analysis (25). FC requires standardization on multiple levels since different instruments give different results, as do variations in the sample matrix, the type of target cells and the particular staining method (34). Overall, the sensitivity of FC may be affected by different variables that are peculiar for the samples, making other methods for diagnosis of *G. lamblia* more sensitive as they are not affected by these variables. Therefore, development of more effective technology is invited to improve the sensitivity of FC. Up to our knowledge this study is the first to be done in Egypt concerning the role of FC in *Giardia* detection in human stool samples and further studies will be needed in attempt to increase the sensitivity of FC either by using more advanced flowcytometers, applying different gating strategies, or employing multiple staining of the *Giardia* cysts.

CONCLUSION

Although direct microscopic examination requires experienced staff, it is more economical and quick in diagnosis of *Giardia* and can detect other parasites; therefore, it should be used as a first choice. In well-equipped laboratories, DIF is an excellent technique for *Giardia* detection. However it could be resorted to suspected cases with clinical manifestations and negative microscopy even after concentration techniques. Though FC has exceptional high speed, and has been suggested as a rapid and sensitive method for screening large numbers of fecal samples for the presence of protozoan cysts; it did not show the expected sensitivity in our study. Further studies will be needed to improve the sensitivity of the FC in detection of *Giardia* cysts in stool by creating an optimized FC protocol that provides an accurate, fast, simple and automated detection method for clinical diagnosis. This may be achieved by using better stool purification methods, improved gating strategy, multiple immune staining of the cysts, and using more advanced flow cytometers. If FC would produce results that are comparable to DIF, it could be an effective alternative method for the detection of *Giardia* cysts, as it has the benefit of speed and would not depend on an experienced microscope viewer.

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