

Full Length Research Paper

Detection, Enumeration and Viability Evaluation of *Giardia* Cysts in Water Samples Using Flow Cytometry

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Giardia lamblia is a protozoan parasite known to be an important waterborne pathogen. The faeco-oral transmission of the parasite leads to the presence of its cysts in the environment. *Giardia* cysts are abundant in surface waters worldwide. Knowledge of the prevalence of *Giardia* cysts in water resources is important for controlling its transmission. The present study was designed for detection of *G. lamblia* cysts, its seasonal variability, count and viability assessment in water supplies of Assiut University Hospitals and Faculty of Medicine; using flow cytometry. Forty eight water grab samples (20 liter per sample) were collected from water supplies of the faculty and hospital's buildings from inlet, outlet and tanks during winter and summer 2014. All water samples were subjected to filtration, elution and concentration followed by flow cytometry. *G. lamblia* cysts were detected in 14 water samples with detection rate of 29.2%. The highest rate of positive samples was recorded in winter. The mean cysts number was 1066.3 cysts/L in summer and 837.1 cysts/L in winter. The percent of viable *G. lamblia* cysts reached 6.2% in summer and 5.75% in winter with insignificant difference. The results of the present study reflected the need for more efficient methods adapted by the water treatment facilities in this locality for controlling the quality of drinking water. The results demonstrated the benefit of flow cytometry as a rapid and simple method for evaluation of *Giardia* cysts in water samples.

Keywords: *Giardia* cysts, Water, Flow cytometry, Egypt.

INTRODUCTION

Giardia lamblia is protozoan parasite that infects the gastrointestinal tract of vertebrate animals, including mammals, birds, reptiles, amphibians. Species within these

genus cause human giardiasis, which constitutes the most common cause of protozoan diarrhea and lead to considerable morbidity and mortality. About 200 million people have symptomatic giardiasis worldwide, with prevalence of 2–5% in industrialized countries and 20–30% in developing regions of Asia, Africa and Latin America (WHO, 2011). Pathologic changes, as well as clinical

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manifestations of this condition, depend on numerous factors, the most important of which are the virulence of *G. lamblia* strain, number of inoculated cysts, age and immune reactivity of the host at the moment of infection (Tasic et al., 2010).

Transmission of giardiasis is typically associated with poor faecal-oral hygiene. Water and food are the most common transmission vehicles, although person-to-person or animal-to-person direct contacts are also important routes of infection. Immune compromised individuals (i.e. AIDS and cancer patients, the very young or the elderly) are most at risk from the clinical consequences of these diseases (Keserue et al., 2011). Water is the major transmission route of *Giardia*, where it can resist and remain infective due to its robust form, the cyst (Thompson, 2000). The infected hosts shed in the environment a large number of this transmissible and infective stage, contributing to an increase of environmental contamination, in particular water sources. Cysts not only remain infective for long periods in environment but are also resistant to the conventional treatment processes of water, representing a serious problem of public health (Castro-Hermida et al., 2009; Lobo et al., 2009). *G. lamblia* has been considered the most common cause of waterborne disease outbreaks since 1971. Outbreaks associated with contaminated drinking water are usually larger than those caused by contaminated recreational water (Smith et al., 2007). The sources of drinking water include rivers, reservoirs, canals, or low land reservoirs. The pathogens can enter these surface waters from agricultural or urban runoff, wastewater treatment discharges or biosolids causing a cycle of infection to humans and animals (Caccio et al., 2003; Hunter and Thompson, 2005).

Giardia cysts are abundant in surface waters worldwide; their concentrations are reported to be in the range of 0.01 to 100 cysts per liter. They are reported to be strongly resistant to disinfection, including chlorination, and difficult to remove by standard filtration (WHO, 2002). They survive for up to 2 months in water at 8°C (Meyer and Jarroll, 1980).

Detection of *Giardia* cysts in water is completely different from that traditionally used for quantification of fecal indicator bacteria in the water industry (Castro-Hermida et al., 2010).

Flow cytometry (FC) is a measurement of characteristic single cells (cyto) suspended in a flowing saline stream (Ormerod, 1990). Flow cytometry has been used in microbiology for detection of multiple organisms (Kamel et al., 1999). It has been used for parasitological studies, e.g. estimation of T lymphocyte subpopulations in schistosomiasis (El Sherbiny et al., 2003) and cytokine profile evaluation (Ferrari et al., 2000). Flow cytometry was used in the diagnosis of *Cyclospora* in fecal samples (Hussein et al., 2007). In Egypt, no continuous routine wide

scale evaluation of water pollution is being done to detect contamination with protozoa (Khalifa et al., 2011). The present study was aimed to evaluate the prevalence, count and viability assay of the environmental stage of *G. lamblia* in drinking water samples in Assiut University Hospitals and Faculty of Medicine using flow cytometry; hence assessing the risk of human infection associated with water consumption in these sites.

MATERIAL AND METHODS

Water samples collection

Forty eight drinking water samples were collected from Assiut University hospitals and Faculty of Medicine drinking water supplies. Water samples were given a code numbers according to the sequences of collection and included: the 3 inlets at main hospital gates which coming from the supplying local water treatment plant (H1-3), outpatient clinics (H4-6), inlets, tanks and outlets of all hospitals and faculty buildings (H 22-24). These hospitals included main hospital building (H 7-9), children hospital (H10-12), woman health hospital (H13-15), Al-Rajhi liver hospital (H16-18) and Urology and Neurology hospital (H19-21). Water samples were collected during December 2013, January, February 2014 (winter season) and June, July, August 2014 (summer season). 20 L volume sample size (Grab sample) was used (Smith and Grimason, 2003). Each sample was collected in clean, sterile screw cap plastic containers labeled with site and date of collection.

Filtration of water samples

Each water sample was passed through a filter apparatus consisted of three filters. The first one had pore size 100 µm to remove large particles and debris, the 2nd one with 25 µm pore size to remove algae and parasites more than 25 µm and the third membrane filter with an equivalent pore size of 1µm (7cm in diameter) supplied by IDEXX Laboratories (New South Wales, Australia).

Elution and concentration of *G. lamblia* cysts

The third membrane filter was eluted according to Pezzana et al. (2000) trials, in which Laureth 12 elution buffer was replaced with phosphate-buffered saline (PBS)-Tween-antifoam buffer (PBS elution buffer) composed of 2L of PBS buffer [PH 7.4], 300 ml of Tween 80 and 300 ml of antifoam.

The membrane was placed in 100 ml PBS elution buffer, on a wrist action shaker for 5 min at 300 rpm. Repeating this step twice more with changing the orientation of the filter in the shaker was done. The elute solutions were collected, centrifuged at 1,100 rpm for 15 min. The

supernatant was carefully aspirated and discarded. Samples were preserved in 2.5% potassium dichromate until used.

Flow cytometry procedure

Positive control sample was provided with the kit (PC-101) with known numbers of the parasite. Negative control sample was boiled distilled water. Positive and negative control samples were used before the tested samples to calibrate the device. Flow cytometry commercial kit labeled Aqua-Glo™ kit (A100FLK, Waterborne, USA) was used according to US EPA method 1623, 2005.

Processing of samples

200 µL of water sample (sediment after membrane filter elution and concentration) was divided into two FC tubes. The first tube was performed as a non-stained sample (auto fluorescence or auto) which served as self-control for each sample to detect nonspecific fluorescence. The second tube was called sample (test). DAPI (4,6-Diamidino-2-phenylindole) staining was performed by adding 50 µL of working dilution which was prepared by diluting 1 µL of DAPI to 5 µL with PBS (PH 7.4) into both tubes, left for 1 min at room temperature away from light. The tubes were rinsed by adding 50–100 µL Sure-Rinse™ wash buffer and centrifuged at 3000 rpm for 1 min at 4°C. The supernatant was carefully aspirated and pellets were used. One drop (approximately 45 µL) of Aqua-Glo™ (antibody reagent) was applied, to the second test tube only. The tubes were incubated at room temperature for at least 35 min in a humid air chamber. The auto tube was rinsed by adding 50–100 µL Sure-Rinse™ wash buffer and centrifuged as previous. The tubes introduced into the flow cytometer (FACS Calibur BD Biosciences, Sydney) as plattlet setting with accusation of 10^5 events in each setting, gate 1 and reading Cy3 as FL2 and DAPI as FL3. It allowed the samples to pass through standard filters; FL2: laser band pass per filter equal 585/42 nm wave length and FL3: 650 nm. Every positive and negative control was analyzed in the same manner to ensure that any fluorescent debris did not appear in the analytic gate.

The optical characteristics of purified cysts were evaluated by Dot plots and histogram. Dot plot which was a graph with 2 axis; one was representing forward scatter (FSC) and other was representing side scatter (SCC). The parasite, which was stained with the fluorochrome stains (Cy3 and DAPI) was detected as dots on this graph according to the cell volume (Forward scatter FSC) and the inner complexity of the parasite (side scatter, SCC). These dots were called events and the region surrounding these dots was called gated region, gate 1(R1). Dot plot, in which each event represented *Giardia* cysts, appeared as FL2

according to the intensity of the fluorescence. It estimated the percentage of the parasites from the total number of particles detected by the device. The percentage of viable parasites stained by DAPI was estimated as FL3 in the dot plot. Histogram was representing *G. lamblia* counted by Cy3 (test) in comparison with the sample self-control (auto) (Khalifa et al., 2011).

Atmospheric temperature

Daily mean of maximum and minimum temperature was acquired from the Egyptian Meteorological Authority, Assiut station which occurs within Assiut University campus. The average maximum temperature for winter and summer in Assiut were 20.3°C and 37°C respectively (Moatamed, 2005).

Statistical analysis

Data were collected, tabulated statistically analyzed using Statistical Package for Social Sciences (SPSS) program version 20.0 software. Categorical variables were described by number and percent (N,%), where continuous variables described by mean and standard deviation (Mean \pm SD). Chi-square test used to compare between categorical variables. While t-test used in comparisons between continuous variables. Pearson correlation coefficient was used to assess the association between continuous variables. P value equal to or less than 0.05 was considered statistically significant.

Ethical consideration: The study protocol was approved by the Research and Ethic Committee of Faculty of Medicine, Assiut University. Also administrative authority's approvals from the officials were obtained to access the drinking water sources in the hospitals and faculty buildings.

RESULTS

In the present study, 48 drinking water samples were collected from Assiut University hospitals and Faculty of Medicine buildings examined by flow cytometry to detect *G. lamblia* cysts. Twenty four samples were collected in summer while the other half was collected in winter.

Results obtained from flow cytometry for each tested water sample were analyzed as in figure 1 and 2 which showed both positive and negative sample's results respectively; expressing each cysts surface antigen detected as events at the gate region 1 (R1) using monoclonal antibodies and fluorochrome stains (Cy3 and DAPI stain for determining parasite viability).

Concerning the prevalence of *G. lamblia* in the tested water samples; 14 water samples of 48 examined samples

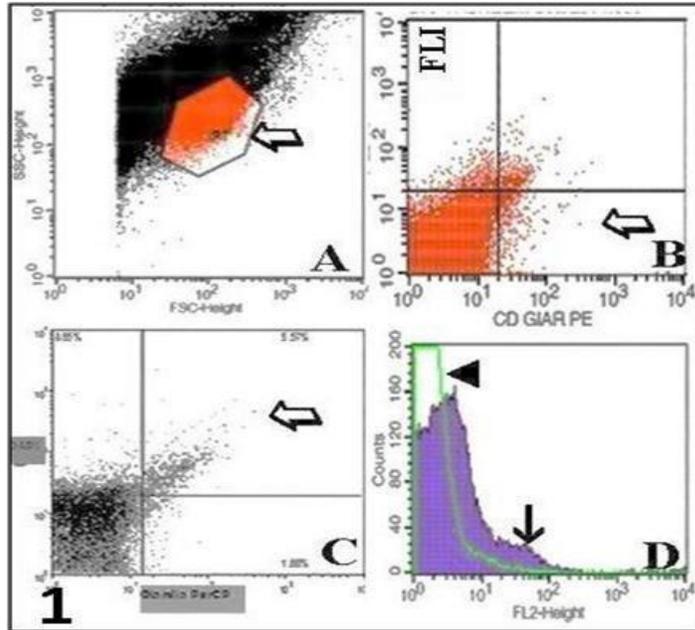


Figure 1: Flow cytometry dot plots and histogram showing positive result for *G. lamblia*: **A:** *G. lamblia* gated region R1 (arrow) showed side-scater (SSC) against forward-scater (FSC), **B:** Events representing *G. lamblia* in the right lower quadrant (arrow) **C:** Viable *G. lamblia* cysts with DAPI stain were detected in the upper right quadrant FL3 (arrow) **D:** Histogram showing a positive sample for *Giardia* represent *Giardia* counted by flow cytometry (arrow) compared with Auto (arrowhead).

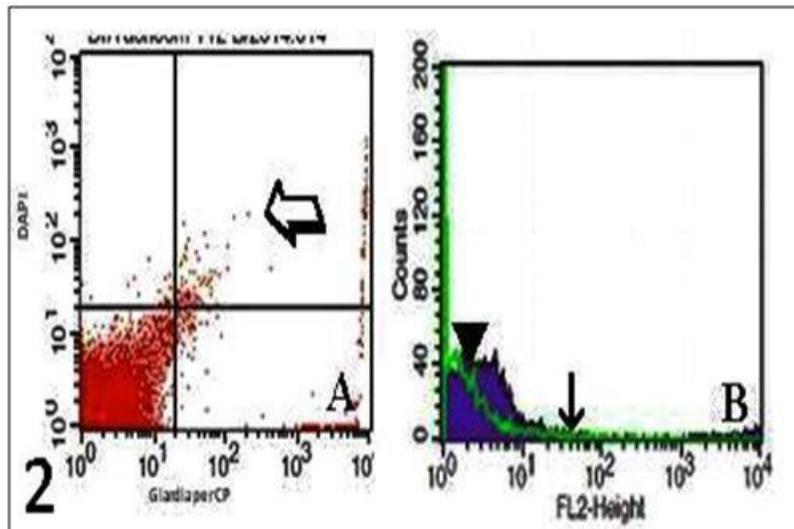


Figure 2: **A:** Flow cytometry dot plot showing negative sample with absence of viable *Giardia* cyst in water samples with DAPI stain in the upper right quadrant (arrow), **B:** Histogram showing a negative sample for *Giardia* counted by flow cytometry (arrow) compared with Auto (arrowhead).

were infected representing 29.2% prevalence rate. Cysts were more prevalent in winter 37.5% than in summer 20.8% but no significant seasonal variation was proved as P value >0.05 (Table 1; Figure 3).

As regard the cyst's count in each examined sample; the intensity of water contamination was higher in summer with

mean cyst's count of 1066.3 events (cysts)/L than in winter with mean cyst's count of 837.1 events (cysts)/L. The higher cysts contamination or count was detected in urology and neurology hospital in winter than other sites, while in summer highest contamination was detected in the out patients clinics. The lowest contamination was

Table1. Comparison of the occurrence of *Giardia* cysts in water samples by seasons.

Season	Number of samples	Number of positive sample	Percentage	P. value
Winter	24	9	37.5 %	0.341 ^{ns}
Summer	24	5	20.8 %	
Total	48	14	29.2 %	

Ns: No significant difference (P>0.05)

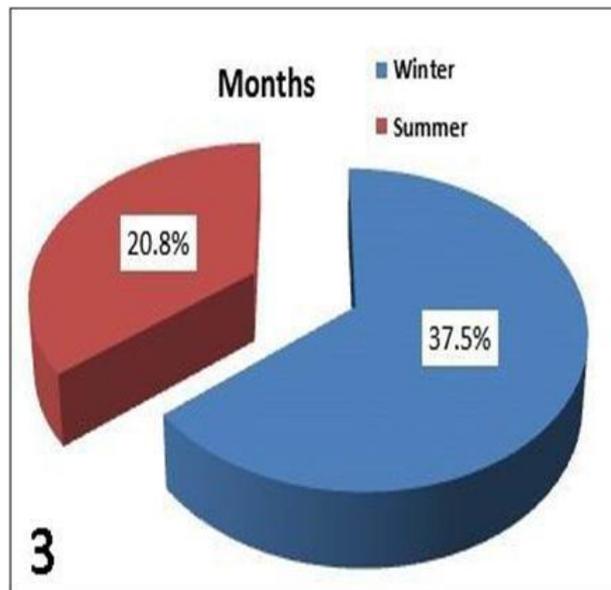


Figure 3: Prevalence of *Giardia* cysts in water samples in relation to the seasons

observed in Al-Rajhi liver hospital in both winter and summer seasons. (Table 2; Figure 4).

DAPI staining had detected the percent of viable cysts in the 48 examined samples. The results showed no significant difference in regard to viability percent between winter (5.75%) and summer (6.2%). Also no statistical correlations were detected between viability percent and atmospheric temperature at summer and weak correlation were observed between viability percent and atmospheric

temperature at winter. The viability percent was observed to increase in winter months (Table 3; Figure5).

DISCUSSION

Several epidemiological and ecological studies have been conducted to detect the presence and the abundance of *Giardia* and *Cryptosporidium* in surface waters (Rose and

Table 2: Mean *Giardia* cysts intensity detected by flow cytometry at different hospitals sites during winter and summer seasons.

Hospital buildings	Means winter cysts/L	±SD	Means summer cysts/L	±SD
Main Inlets From water plant	0.0	0.0	655.0	1134.5
Out patients clinic	886.7	1535.8	1970.7	3413.3
Main Hospital	1504.0	1327.4	1210.7	2096.9
Children hospital	612.3	1060.6	0.0	0.0
Woman health hospital	1997.3	1742.3	1193.3	2066.9
Al-Rajhi Liver Hospital	123.3	213.6	0.0	0.0
Urology&Neurology hospital	2444.8	306.0	0.0	0.0
Faculty of Medicine	962.3	380.3	1666.7	2886.8

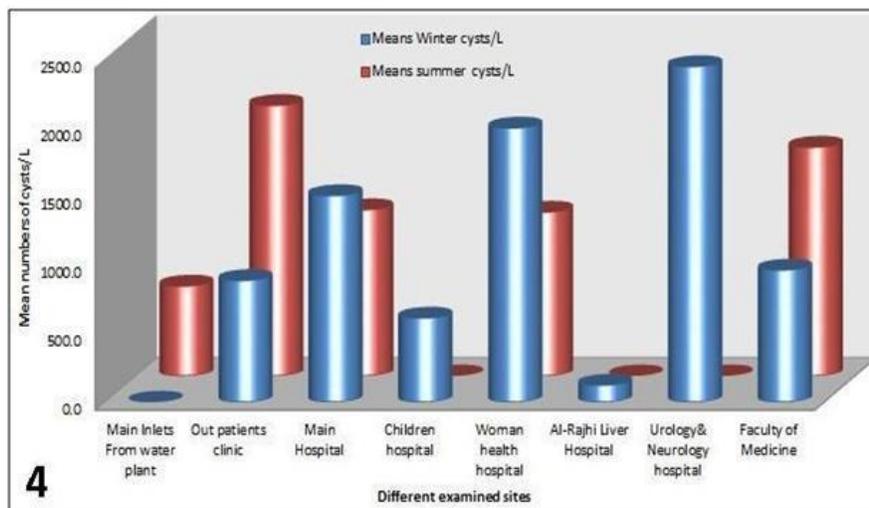


Figure 4: Mean cysts intensity detected by flow cytometry at different examined sites during winter and summer seasons.

Table 4. Seasonal variability of *G. lamblia* in term of prevalence, intensity (number of events/100 µl /L) and viability detected by flow cytometry.

Hospitals Buildings	Sample place	Sample code	Winter				Summer			
			Temp. in °c	cysts no / 100 µl	cysts no / L	Viability per. %	Temp °c	cysts no / 100 µl	cysts no / L	Viability per. %
Main Inlets From water plant	Main pipe outlet	H1	22	0	0	0	37	0	0	0
	Injury entry gate	H2	22	0	0	0	37	786	1965	1.3
	Outpatient clinics gate	H3	22	0	0	0	38	0	0	.0
Outpatient clinics & Residents Rest Room	Inlet	H4	21	1064	2660	1.6	39	0	0	0
	Tank	H5	18	0	0	0	39	0	0	0
	Outlet	H6	18	0	0	0	39	2364.	5912	6.2
Main Hospital	Inlet	H7	17	1004	2512	5.75	40	0	0	0
	Tank	H8	17	800	2000	3.1	40	0	0	0
	Outlet	H9	17	0	0	0	40	1452	3632	1.12
Children Hospital	Inlet	H10	19	0	0	0	39	0	0	0
	Tank	H11	19	0	0	0	39	0	0	0
	Outlet	H12	19	734	1837	1.4	39	0	0	0
Woman Health Hospital	Inlet	H13	20	1114	2787	1.12	40	0	0	0.31
	Tank	H14	20	1282	3205	1.3	40	0	0	0
	Outlet	H15	20	0	0	0	40	1432	3580	1.43
Al-Rajhi Liver Hospital	Inlet	H16	20	0	0	0	38	0	0	0
	Tank	H17	21	148	370.0	0	38	0	0	0
	Outlet	H18	21	0	0.0	0	38	0	0	0
Urology & Neurology Hospital	Inlet	H19	22	1086	2715.0	0.38	38	0	0	0
	Tank	H20	22	1002	2507	1.5	39	0	0	0
	Outlet	H21	22	845	2112.5	2.03	39	0	0	0
Faculty of Medicine	Inlet	H22	23	242	605.0	0.22	39	2000	5000	1.7
	Tank	H23	23	368	920	1.20	40	0	0	0
	Outlet	H24	23	544	1362	1.5	40	0	0	0

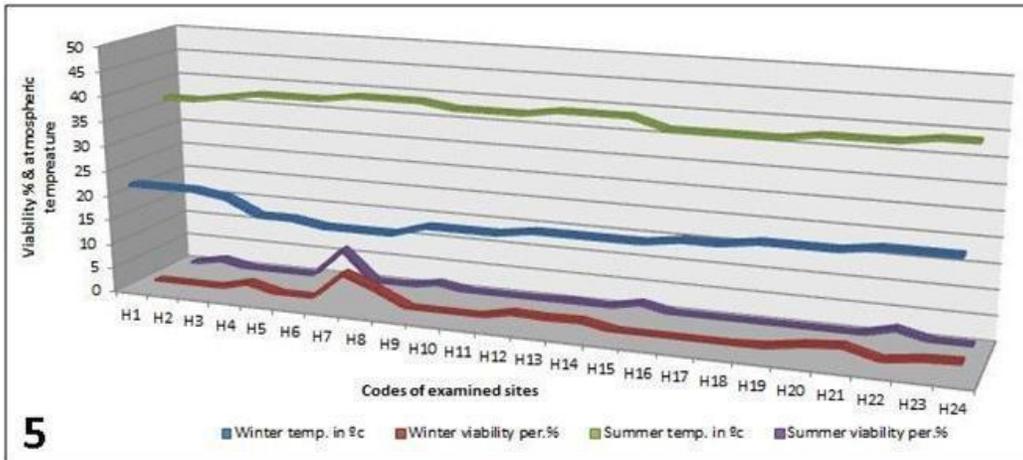


Figure 5: Correlation between *G. lamblia* viability % and atmospheric temperature at time of samples collection using flow cytometry.

Slifko, 1999; Barwick et al., 2000; Sroka et al., 2013). As drinking water supplies are most frequently obtained by the collection and treatment of surface water, it is important to constantly monitor the presence and seasonal fluctuation of these parasites in a given catchment area (Sroka et al., 2013). There is no mandatory local or national monitoring or surveillance programs for pathogenic protozoa in surface waters in Egypt. Only sporadic studies have been done on different sources of water and there are no actual studies of the prevalence of these protozoa in water in Egypt (El Shazly et al., 2007).

Currently available methods for detection of *Giardia* cysts in water are at best tentative; and recovery is low and variable (Khalifa et al., 2011). These methods include microscopy, immune fluorescent techniques and ELISA. On the whole, detection methods need to be evaluated especially for low and accurate counts of cyst number (Castro-Hermida et al., 2010). Finding cysts in water is not necessarily significant, since some of the detected organisms may be nonviable and thus pose no threat to public health. Consequently, there has been considerable interest in using methods capable of determining cyst viability (Dechesne and Soyeux, 2007). Conventional detection relies on cyst concentration, isolation, and confirmation by immune fluorescence microscopy (IFM), resulting in low recoveries and high detection limits (Keserue et al., 2011). Many molecular identification methods based on PCR have been proposed, with detection limits ranging between 1 and 10^5 cysts but difficulties due to small amounts of target DNA, the

presence of inhibitor substances, and false positive results were reported (McIntyre et al., 2000). Different detection methods using flow cytometry (FC) were proposed, employing different staining methods and resulting in variable recovery (Hsu et al., 2005). Flow cytometry is a fast screening detection assay involving the filtration of cysts, concentration and flow cytometric detection. This rapid and robust method can be applied to different water samples and has the prospect of being applicable to various matrices and to the automation potential of flow cytometry (Keserue et al., 2011). Flow cytometry with fluorescence-activated cell sorting (FACS) is used increasingly as an alternative method for separation and enumeration, but difficulties are encountered when auto fluorescent algae are present and cross-reactions of the MAbs lead to unspecific binding (Delaunay et al., 2000). This problem was addressed by Ferrari and Veal, (2003), who developed a two-color detection-only assay for *Giardia* cysts leading to no false-positive results but also having a moderate recovery of 39% for a seeding level of 90 ± 28 cysts in 10 liters of backwash water. The filter apparatus used in the present study consisted of a prefiltration filtration step in order to eliminate large particles and contaminants. Each water samples was filtered through a 1 μ m membrane. This modification was somewhat similar to these done by Keserue et al. (2011) in their study. In this prefiltration filtration step; during sample analysis; cysts and particles were separated and not deposited together on the basic membrane surface this rendered flow cytometric detection and enumeration seemed to be less

prone to signal hiding. The cysts were resuspended in 5 ml PBS and labeled with a specific CY3-coupled surface antibody. These were in agreement with Harba et al. (2012) who concluded that dilution of the samples was essential to reduce concentration of debris thereby making it easier for the antibodies to bind to their target. Even more importantly, it was essential to clear samples of large particle's debris that might clog the apparatus. Therefore, the use of gauze rather than paper filters prior to examination of samples was important and resulted in faster and efficient outcome (Le Chevallier et al., 1991). Also the detection method applied for this study had an adequacy to determine the species and viability of detected cysts.

In the present study, using flow cytometry, the detection rate of *Giardia* cysts was 29.2% (14/48). These results agreed with a study done in Alexandria in 2000, in which *Giardia* cysts were reported in 30.5% of tank water samples (Khalifa et al., 2011). The detection rate in the present study was lower than the detected rate in Alexandria (36.7%) in 2011 in summer season. In other governorates in Egypt, few studies were published on the same subject. The most alarming was the prevalence of *Giardia* positive samples reported in a study done in a Nile delta village more than 20 years ago; comparing Zir water and tap water, where the researchers found that each contained 36% positive samples (Hussein et al., 2007). In Dakahlia, a study carried out in 2007 detected *Giardia* in portable water samples with a rate of 2.1% (El Sherbiny et al., 2003). Also *Giardia* cysts were detected in 33% of tap water in El Hawamdia (Egypt) and 50% in Meet Fares (Egypt) using PCR (Khalifa et al., 2011).

In studies done around the world, *Giardia* cysts were detected in Argentina in 31% of drinking water sources (Smith and Grimason, 2003). In 2002, monitoring a water plant for one year in Japan using coagulation- flocculation, sedimentation and rapid filtration, the investigators detected *Giardia* in 92% of raw water samples and in 12% of filtered samples (Pezzana et al., 2000). In a survey in Sweden, *Giardia* cysts were detected in 26% of water samples (US EPA method 1623, 2005). However, lower detection rates were reported in USA in 1991 using a combined immune fluorescence test, the investigators found *Giardia* in 17% of filtered water samples (Le Chevallier et al., 1991). Lower detection rate was also reported in Russia from different water samples collected during 2006; where *Giardia* cysts were found in only 9.6% of these samples using immune fluorescence test (Karanis et al., 2006).

The frequency of positive water samples was more in winter 37.5% than in summer 20.8% but with no significant seasonal variation. This seasonal trend seems to be related to the stronger resistance of cysts in low temperature. These results agreed with that of a study done in Korea, where *Giardia* cysts were more frequently

found in winter in drinking water supplies (De Regnier et al., 1989). The intensity of water contamination was estimated by the quantity of Cy3 staining ability of the parasite and expressed as total events taken by the flow cytometry device in all samples. Although there was a higher detection rate of *Giardia* cysts in winter months than in summer; the intensity of water contamination in the present study was higher in summer; with mean cyst's count of 1066.3 cysts/L; than in winter; with mean cyst's count of 837.1 events (cysts)/L. These findings could explain the higher prevalence of human infection with *Giardia* from mid-summer to early fall than in winter (Furness et al., 2000). Swallowing as few as 10 cysts might cause someone to become infected and ill (CDC, 2014). Not only the number of *Giardia* cysts in water samples which determine the potential infectivity of this water source but also the more important is the viability rate of the detected cysts in the examined samples which played an important role in the transmission cycle of giardiasis (Harba et al., 2012). In the present work; DAPI staining had detected the percent of viable cysts in the 48 examined samples. The results showed no significant difference in regard to viability percent between winter and summer. The viability percent was observed to increase in winter months. These findings suggested that the transmission of giardiasis in the examined locality was more dependent on cyst's count. An advantage gained by flow cytometry was its ability to yield a perspective of the parasitic load, including viable and dead forms, in each sample. The 1st histogram detected the percentage of the parasites and was followed by the 2nd one which detected their viability. Accordingly, flow cytometry is able to quantify the parasites in water samples (Hsu et al., 2005). The importance of this information lies in that; this parasite has to be present in a certain load to cause illness (Adam, 2001).

CONCLUSION

The present study confirmed the presence of viable *Giardia* cysts in high numbers in drinking water supplies of the examined sites making people more exposed to the risk of acquiring the infection. These results revealed the need for efficient routine monitoring methods adapted by the water treatment facilities in this locality for water pollution by *G. lamblia* cysts for controlling the quality of drinking water. The results of the present study also clearly demonstrated that; flow cytometry as a rapid, simple and accurate technique can be used in estimating the quantity and viability of the parasites in water sample. Flow cytometry can be potentially used for routine monitoring the cysts in water. These advantages have a valuable application in health services.

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