Int.J.Curr.Microbiol.App.Sci (2019) *8*(12): 2263-2276

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 12 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.812.269

Prevalence of *Giardia intestinalis* and *Cryptosporidium parvum* Parasites in Drinking Water in Menoufia Governorate, Egypt

Dalia Shafey[1], Mohammed M. Aboamer[2], Karema Abd Elhady Diab[3], Heba Mohamed Abdallah[4], Marwa F. Yousef [5] and Marwa Ahmed Gouda[1]*

[1]Department of Clinical and Molecular parasitology, National Liver Institute (NLI), Menoufia University, Shebin Al Koom, Menoufia, Egypt
[2]Manager of Biology Lab at Tala Holding Company of Water, [3]Department of Clinical Pathology, [4]Department of Epidemiology and Preventive Medicine, National Liver Institute(NLI), Menoufia University, Shebin Al Koom, Menoufia, Egypt

**Corresponding author*

Keywords

Cryptosporidium, Giardia, Prevalence, Drinking water, Egypt

Article Info

Accepted:
17 November 2019
Available Online:
10 December 2019

A B S T R A C T

Entero-parasites protozoan of the genera *Cryptosporidium* and *Giardia* have emerged over the past decades as major waterborne pathogens with an increasing number of outbreaks reaching over one hundred and sixty. In line with the national goals of providing safe drinking water supplies, this research was designed. We aimed to estimate the prevalence of *Cryptosporidium parvum* and *Giardia intestinalis* infections in surface water plants from ShebinAl-Kom and Tala, Menoufia Governorate and to evaluate routine water purification methods used for parasitic elimination. The following methods were used for assessment of samples; mechanical filtration, staining techniques and qPCR assay for detecting DNA of (oo) cysts n water samples.Water samples collected over one year (four seasons) were 87 samples. Microscopic examination (our golden test) revealed, 49 samples positive for *cryptosporidium*, fourteen positive samples for *Giardia* and four samples were positive for both of them whereas, qPCR revealed only eight positive samples for *Giardia* and ten positive samples for *cryptosporidium*. A statistically significant result concerning seasonal variation was documented where water contamination crested in Summer ($p < 0.05$). The intensity of infection decreased significantly after water treatment in both Shebin and Tala water stations (P= 0.01).From the present survey, we found that the prevalence of giardiasis and cryptosporidiosis accounted for 29.8% and 47.1% in Shebin Al-Kom and Tala respectively. The parasitic cyst wall structure rendered diagnosis by qPCR. Genotyping of water samples is recommended for sourcing of infection which may be accused of water-borne outbreaks.

Introduction

Two billion people at least are estimated worldwide, by World Health Organization (WHO), for the consumption of polluted water and one hundred and forty four millions depend on surface water for their drinking supply. Many diseases are transmitted through water contamination with 485 thousand yearly live loss (WHO, 2019).

Giardia intestinalis (*G. intestinalis*) and *Cryptosporidium parvum* (*C. parvum*) are protozoan parasites that are responsible for widespread gastrointestinal diseases. Some authors reported that water was the main source of about ninety per cent of reported outbreaks by these protozoans, while about 10% were related to food contamination and person-to-person contact (Heitman *et al.*, 2002), the reported frequencies of surface water contamination with *Giardia* and *Cryptosporidium* were from 60 to 96% in the United States (Nichols *et al.*, 2003) and from 20 to 64% in Canada (Daley *et al.*, 2018).

Symptoms of infections range from abdominal cramps, headache, nausea, vomiting, low-grade fever to life-threatening watery diarrhea. The first symptom may appear two to ten days after infection and may last for about two weeks. However, in some individuals, the condition may worsen after recovery. Medical treatment shortens the illness and 50% of adults get free of infection within one to three months without treatment. It is very important to determine the number of viable(capable of infection) *Cryptosporidium* oocysts and *Giardia* cysts to assess the state of infectivity (Chalmers and Davies, 2010).

Incidence of water-borne outbreaks caused by these pathogens was usually underestimated due to their small size and frequently low number excreted in water samples, and the need for well-trained experienced personnel to detect of *Cryptosporidium* oocysts and *Giardia* cysts. Therefore, many efforts were made to improve detection methodologies through different techniques such as flow cytometry, laser scanning, immunomagnetic separation, and PCR (Pollock *et al.*, 2008).

Quantitative real-time PCR (qPCR) was applied in the past few years as it provides a great sensitivity in detection plus the ability to estimate quantity of different parasites (Robertson and Gjerde, 2001; Xiao and Fayer, 2008).

In particular, PCR is an attractive diagnostic procedure as being rapid, sensitive, and pathogen-specific. While many PCR methods have been described for both *Giardia* and *Cryptosporidium* detection, this technology was slowly emerging as a practical method for pathogen assessment for water quality.

Which may owe to low numbers of cysts and oocysts in water and the requirement of significant sample concentration to reduce large volumes to the quantities suitable for PCR.

The new generation of PCR methods could detect *Cryptosporidium* and *Giardia*species that are infective to humans (Xiao and Fayer, 2008).

The ability of *Giardia intestinalis* and *Cryptosporidium parvum* to cause waterborne disease is well documented (Wright *et al.*, 2018) However, widely used analytical methods for detecting the presence of these parasites in water didn't provide the quality of data required to assess health risk and effective management of this problem (Weintraub, 2006). Since immune-compromised patients, those with cancer, AIDS, old or very young people are more liable to infection and exaggeration of symptoms and the studied parasites are of

great concern in developing countries as many governorates in Egypt rely on surface water as a drinking source, therefore it was mandatory to study the prevalence of waterborne protozoa, namely *Giardia & Cryptosporidium*, and to give more information which may be lacking in Menoufia Governorate, Egypt.

Materials and Methods

Study design

Herein, a cross-sectional study was carried out. Water samples were collected from Shebin Al-Kom and Tala water holding companies, (before, during and after water processing) as representative main centers for water purification in Menoufia Governorate from which health quality samples for the whole Governorate are present over a year from July 2017 to July 2018.

Survey protocol

Eighty-seven water samples were collected and they were subjected to the following techniques for assessment.

Mechanical filtration of water samples (about 10 L volume); to maximize (oo)cysts recovery and ensure are presentative sample(Pollock *et al.*, 2008).

Staining with iodine, modified Zeihl Neelsen stain and specific fluorescent antibodies 4′,6-diamidino-2-phenylindole(DAPI stain); to show the viability of cysts and oocysts.

Counting parasites using differential interference contrast microscopy bySEDGEWICK –RAFTER 50 cell (S.R) slides**.**

Detection of the parasites by Real-Time PCR (Xiao and Fayer, 2008)**.**

Sample collection

Water samples were collected from Shebin Al-Kom and Talawater holding companies, the samples were ten litter each, in a clean labelled plastic container and were mixed with 10 ml of Na thiosulfate solution (made by adding 3.977 gm of Na thiosulfate with one litre of distilled water) (Environmental, 2002)**.**Samples were collected every week for the whole year except in winter season where samples were collected nearly every two weeks due to the cold phase stage present in water which renders the presence of organisms in this period (15 samples from both cities were collected through whole the winter season).

Sample processing

The samples were filtered by a stainless steel filtration unit with a pump, through a membrane filter (cellulose nitrate filter) with pore size 0.45 µm.

Parasitological examination

Parasitic counting

The concentrations of cysts and oocysts stocks were determined with the S.R slide. The cell holds 1000 cubic ml of liquid one ml depth over an area of 50x20 ml. The bases were divided into one ml squares.

A cover glass was used to trap liquid into the correct depth. By observing the liquid through a low magnification microscope, objects contained in each cubic ml were identified and counted.

Sample staining

All samples were stained by Lugol's iodine, modified Zeihl-Neelsen, and DAPI stain to assess parasitic viability.

Microscopy

Microscopic examination was performed to detect parasites after staining with iodine and Z.N stains. Slides stained with vital stain, DAPI, (Cat No. GTX16206)were examined using a Zeiss Axioskopepi fluorescence microscope fitted with x 10 eyepieces and x 25 oil and x 100 oil Plan Neofluar objectives, with dichroic mirror and filters for FITC (blue 09), PI (green 15) and DAPI (UV 02). Slides were examined for the presence of (oo)cysts where *Cryptosporidium* oocysts appeared as spherical shapes with an apple green fluorescence of its wall and a diameter of 4-7 µm and *Giardia* cysts appeared as oval to spherical shapes with an apple green fluorescence of the wall and a size of 6-15 x 7-18 µm(Weintraub, 2006).

Molecular diagnosis by qPCR

*DNA extraction

Principle and procedure

The DNeasy Power Water Kit (Qiagen) (Cat No. 14900-50-NF) began with filtration of a water sample onto a filter membrane which is then added to a special 5 ml bead beating tube containing a unique bead mix. Rapid and thorough lysis occurred through vortexing in a specially formulated lysis buffer that enhanced the isolation of microorganisms from the filter membrane. Freezing and thawing process was done by putting the filtered water samples in liquid nitrogen for two min then transferred to boiling water for two minutes, this process was repeated for five cycles. This step is important to cause the rupture of *Giardia* cysts and *Cryptosporidium* oocysts to liberate their DNA contents before DNA extraction to get perfect results (Zhang *et al.,* 2013)**.**

After the protein and inhibitor removal steps, total genomic DNA was captured on an MB Spin Column. High-quality DNA was then washed and eluted from the MB Spin Column membrane for use in downstream applications including PCR and qPCR, according to the manufacturer's protocol the samples were stored at -20ºC until further PCR reactions could be performed (Zhou *et al.,* 2003)**.**

*qPCR

PCR was carried out using the advanced PCR kit (Genesing Handbook HB04.03.05). Principles of Real-time PCR for *C.parvum* and *G.intestinalis*_A-F, specific primer and probe mix was provided and that could be detected through the FAM channel. The primer and probe mix provided exploits the TaqMan® principle. During PCR amplification, forward and reverse primers were hybridized to the *C.parvum* and *G.intestinalis*_A-F DNA**.** A volume of up to 5 µl of DNA was used. The Amplification protocol consisted of two minutes of incubation at 95°C(for enzymatic activation), followed by 50 cycles of alternating temperatures of 95°C for 10 s(for denaturation) and 60°C for one min. (for annealing and extension). A fluorogenic probe was included in the same reaction mixture which consisted of a DNA probe labelled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe was cleaved and the reporter dye and quencher were separated. The resulting increase in fluorescence was detected on a range of qPCR platforms(Xiao, 2010).

Results and Discussion

Water has a vital role within the transmission of numerous distinctive pathogens such as microbes, and parasites, including *Cryptosporidium* and *Giardia*, which are accused of waterborne flaring up of infection (Mons *et al.,* 2009). Prevalence of *G. intestinalis* and *C. parvum* in water samples was evaluated herein. The presence of *Giardia* cysts and *Cryptosporidium* oocysts concerning

the studied geographic areas was assessed by using the microscopic examination of stained specimens (with iodine and modified Zeihl-Neelsen stains). Out of 87 water samples prevalence for *Giardia*sis and cryptosporidiosis was 29.8% and 47.1% in Shebin Al-Kom and Tala respectively.

Five positive samples for *Giardia*sis in Shebin Al-Kom and nine positives for *Giardia*sis in Tala which represented 35.7% and 64.3% of positive cases for *Giardia*sis. While20 specimens were positive for cryptosporidiosis in Shebin Al-Kom, 29 specimens showed positivity for cryptosporidiosis in Tala with 40.8% and 59.2% respectively. Statistical analysis showed a non-significant relation between positive samples and the studied areas ($P>0.05$) (Table 1).

Hamdy *et al.,* (2019) in their research on *Cryptosporidium* and *Giardia* assessment in Beni-Suef potable water mentioned the prevalence of those two parasites in different Governorates in Egypt. While *Giardia*sis infection accounted for 36.7%, 50%, 33%, 13.7%, 13% in Alexandiria, Abo El- nomros, Al Hawamdia, Fayoum, and Gharbia Governorates respectively, cryptosporidiosis prevalence accounted for 100%, 50%, 52,6%, 12.5%, 7.5% in Alexandiria, Assuit, Fayoum, Elminia, and Gharbia respectively (Hamdy *et al.,* 2019). Similar results were reported worldwide where, *Cryptosporidium* was detected in 51% and 25%, while *Giardia* was detected in 0.62% and 2.4% of tap water samples in Jeddah and Makkah respectively (Zakai and Barnawi, 2014). On the other hand, in Iran FeizHadad and his colleagues detected 0% of both parasites in filter system household tap water samples (Feiz Haddad *et al.,* 2016). In Spain, both parasites were detected in 26.8% of examined water samples (Carmena *et al.*, 2007).

Studied parasites were counted at different sampling points of water by S.R slide, the number of *Giardia* and *Cryptosporidium* parasites respectively in raw water samples was 35 (79.5%) and 200 (50.8%) in Shebin Al-kom and 45 (60%) and 180 (60%) in Tala. After exposure of water to treatment the numbers decreased to reach four (9%) and 64 (16.2%) for *Giardia* and *Cryptosporidium* parasites respectively in Shebin Al-kom and 10 (13.3%) and 30 (10%) in Tala. Finally, potable water revealed 5 (11.5%) & 130 (33%) in Shebin Al-kom and 20 (26.7%) & 90 (30%) *Giardia* and *Cryptosporidium* parasites respectively in Tala. A highly significant observation was documented when raw and treated water was compared considering parasite intensity in Shebin Al-Kom station ($P\leq0.01$). From our results it was noticed that the number of parasites decreased in treated water samples then the parasitic number increased again in potable water samples with a significant difference between the two studied stations when raw and treated water were compared with $P=0.01$. This might raises alarm for a hidden possible route for parasite transmission after successful treatment of water in water purification plants such as contaminated water pipes (Table 2 and Figure 2).

Raw water presented the highest percentage of contamination in our work in comparison to other sampling points. This was in agreement with El-Kowrany and his colleagues, who conducted a similar study in Gharbia Governorate, Egypt and found that the most contaminated sampling points were the raw samples (El-Kowrany *et al.,* 2016). Also, a similar result documented by Antonios *et al.,* (2001), in Dakahlia Governorate, detected the presence of *Giardia* and *Cryptosporidium* in potable water samples in 2.1% and 3.1% respectively which make water unsafe for human consumption. Similarly, some studies have found that the positive samples were

often found in raw water samples; for example, in Norway, a survey reported a prevalence of positive parasitic water samples in 16.5% for *Cryptosporidium* and 11.5% of *Giardia* (Robertson and Gjerde, 2001).

The positivity of all drinking water for *Giardia*sis and cryptosporidiosis in high percentages was reported by other authors (Shortt *et al.,* 2006).

Quantitative real-time PCR has proven itself to be a faster and a sensitive approach for the detection and enumeration of microorganisms in various environmental samples. A comparison between qPCR method and microscopic examination (after staining)was performed. The highest number of positive samples for *Cryptosporidium* was observed in raw water 25 samples (51.0%) whereas, their positive samples detected by PCR were only two (20.0%). Also, in *Giardia*ten positive samples were detected in Raw samples (71.4%), however, positive samples detected by PCR were five only (62.5%) (Table 3 and Figure 1).

Our results stated that the number of positive parasitic samples detected by PCR were lower than those detected by microscopy(after staining).These results were in contrast to the finding of Xiao *et al.,* (2006) and Nichols *et al.,* (2003) who reported that the number of positive cases detected by PCR was higher than those detected by microscopic examination.

The fact that the parasitic stages (oo) cysts could stay viable for many months as they are resistant to typical disinfectants(Xiao and Ryan, 2004) and are strong acid resistance because of their specific cell wall structure, as they are covered by thick walls measuring 0.3 to 0.5µm and composed of an outer filamentous layer and an inner membranous layer which is known to be highly resistant to mechanical and chemical disruption (Harris and Petry, 1999) might explain such result. Another possible reason was the failure of complete DNA extraction owing to the need of an increased number of cycles required for freezing and thawing method therefore the DNA remained intact inside the (oo)cysts. Freezing and thawing method was carried out in our work in guidance with Zhang *et al.,* (2013) protocol, five cycles were done for one min in liquid nitrogen and two min in 60c per cycle. On the other side, Nichols *et al.,* (2003) extracted DNA after ten freezing-thawing cycles which consisted of one min in liquid nitrogen and two min in 60c per cycles. This 12- month survey had shown that contamination of water with *Cryptosporidium* and *Giardia* (oo)cysts was present throughout the year in surface water (except winter season), with the highest frequency of occurrence reported during the Summer season with 44.9%for *C.parvum* and 50.1 % for and *G.intestinalis*. A second peak for *Cryptosporidium* was recorded during the Spring, whereas, *Giardia's* second peak was recorded during the Autumn with a statistically significant difference regarding seasonal variation ($P< 0.05$) (Table 4). These results were in agreement with that obtained by El-Kowrany *et al.,* (2016)who did a similar study in Gharbia Governorate. Also, similar results were obtained by Siyadatpanah *et al.,* (2018), they studied *Giardia*sis distribution in Iran.

Climate has a strong impact on the occurrence of protozoan parasites as contaminants in drinking water, especially with extreme weather events. A meta-analysis performed by Young *et al.,* (2014) indicated that the likelihood of the contamination of fresh surface water with *Cryptosporidium* and *Giardia* (oo) cysts was significantly increased during extreme weather events, and particularly surface water sources were more liable to contamination during the monsoon season.

Table.1 Prevalence of *Giardia* and *Cryptosporidium* in relation to geographic areas showing presence of parasites in two localities with the total percent of *Giardia*sis and cryptosporidiosis in relation to the eighty seven examined water samples

Geographic Areas	Positive for *Giardia*		Positive for *Crypto*		Positive for both		Total		χ^2	P. value
	No.	(%)	No.	(%)	No.	(%)	No.	%		
Shebin Al-Kom	5	35.7	20	40.8	1	25	26/87	29.8	2.2	**>0.05**
Tala	9	64.3	29	59.2	3	75	41/87	47.1		
Total	14	100	49	100	4	100	67/87			

Table.2 Demonstrate the results of parasitic counting by S.R slide in relation to sampling point in both water stations

	Shebin Al-Kom Sampling point			Tala Sampling point			χ^2	P
	Raw	Treated	Potable	Raw	Treated	Potable		
Giardia								
No.	35	4	5	45	10	20	6.45	≤0.05
%	79.5	9	11.5	60	13.3	26.7		
Cryptosporidium								
No.	200	64	130	180	30	90		
%	50.8	16.2	33	60	10	30		
Statistical analysis								
P1; Comparison between Shebin and Tala regarding Raw and Potable Water							2.12	0.14
P2; Comparison between Shebin and Tala considering Raw and Treated Water							6.09	**0.01***
P3; Shebin Station: Comparison between Raw and Treated (Highly Significant)							Z test 32.0	P value ≤ 0.01
P4; Shebin Station: Comparison between Potable and treated.							0.1	**>0.05**
P5; Shebin Station: Comparison between Raw and potable.								**>0.05**
P6; Tala: Raw and Treated, Raw and potable and Potable and Treated water								**>0.05**

*Refer to highly statistically significant P value.

Table.3 Comparison between results of PCR and Microscopic examination in relation to the type of water samples

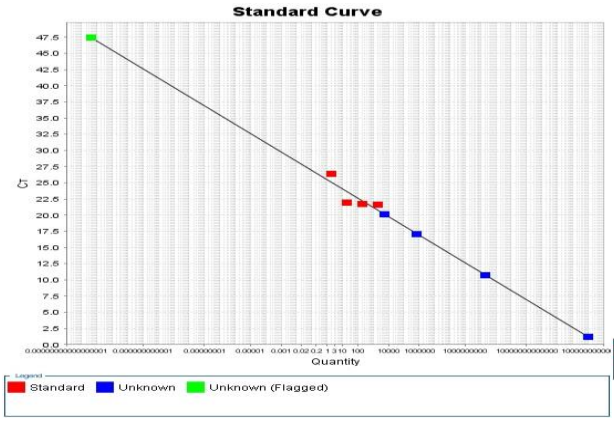
Sample	Microscopy			PCR Results		
	Both	***Cryptosporidium***	***Giardia***	***Giardia***	*Cryptosporidium*	
Raw	1 (25)	25(51.0)	10 (71.4)	5 (62.5)	2 (20.0)	
Treated	2 (50)	4 (8.2)	0 (0.0)	0 (0.0)	4 (40.0)	
Potable	1 (25)	20(40.8)	4 (28.6)	3 (37.5)	4(40.0)	
	4	49	14	8	10	

Table.4 Distribution of infection detected in positive samples examined by Z.N and Iodine Stains for *Giardia* and *Cryptosporidium* in Relation to the Seasons

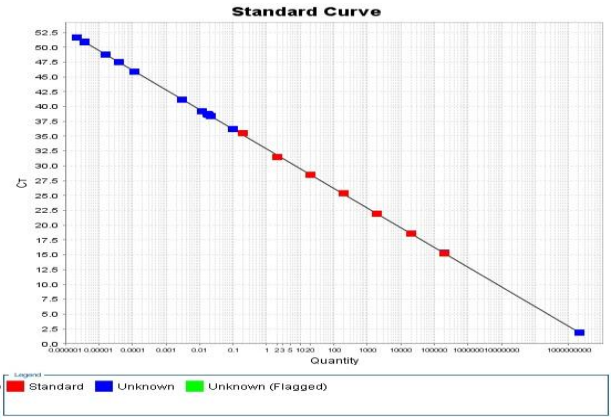
	Season					χ^2	**P**
	Summer	**Autumn**	**Winter**	**Spring**	**Total**		
Giardia							
No.	7	6	0	1	14	28.1	<0.05
%	50	42.9	0.0	7.1	21.0		
Cryptosporidium							
No.	22	9	2	16	49		
%	44.9	18.4	4.0	32.7	73.1		
Both parasites							
No.	4	0	0	0	4		
%	100	0.0	0.0	0.0	5.9		
Total							
No.	33	15	2	17			
%	49.2	22.4	3.0	25.4			

Fig.1 Showing detection of qPCR results for detecting *G. intestinalis* and *C. parvum* in water samples. The standard curves (A, B, C & D) and the amplification plots (E & F) were presented

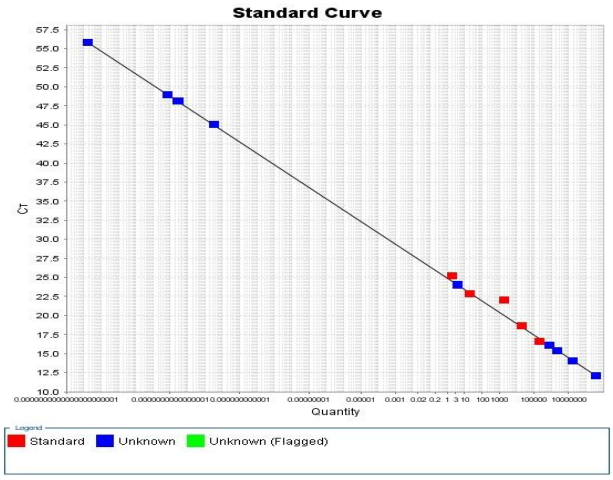
A; Run 1 for *G. .intestinalis*



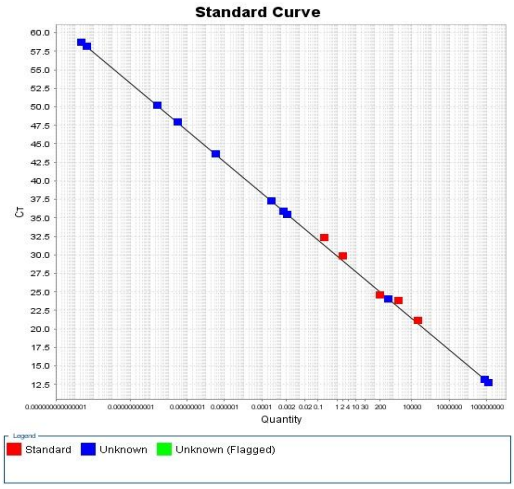
B; Run 1 for C. *parvum*



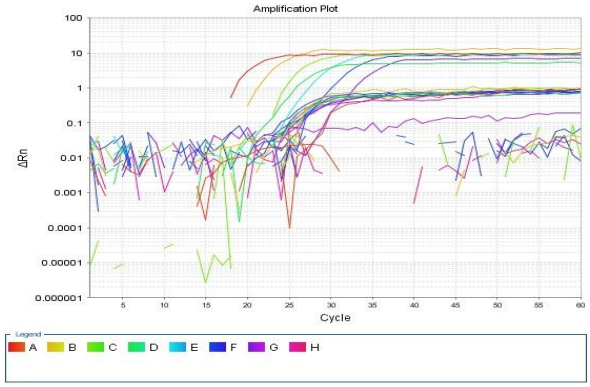
C; Run 2 for *G .intestinalis*



D; Run 2 for *C. parvum*



E; Run 1 for *G.intestinalis*



F; Run 1 for *C. parvum*

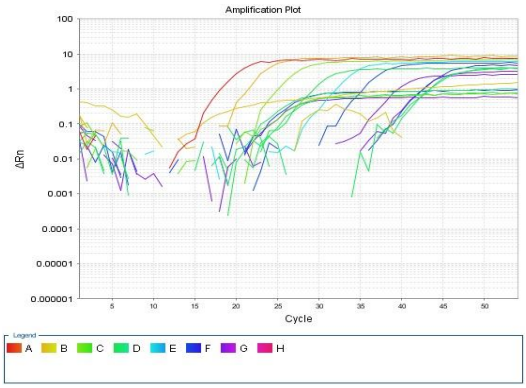


Fig.2 Show *Cryptosporidium* and *Giardia* with different stains. A; Showing *Cryptosporidium* oocysts appearing as spherical shapes with apple green fluorescence in its wall by using fluorescent microscopy. B; *Cryptosporidium* is shown under S.R slides used for counting the parasite under light microscopy (x1000).C; Wet mounting for water samples showing *Giardia* cyst by light microscopy (x1000). D; Wet mounting for water samples showing *Cryptosporidium* oocysts by light microscopy (x1000). E &F; shows *Cryptosporidium* oocysts stained by modified Ziehl Nelseen stain (x1000)

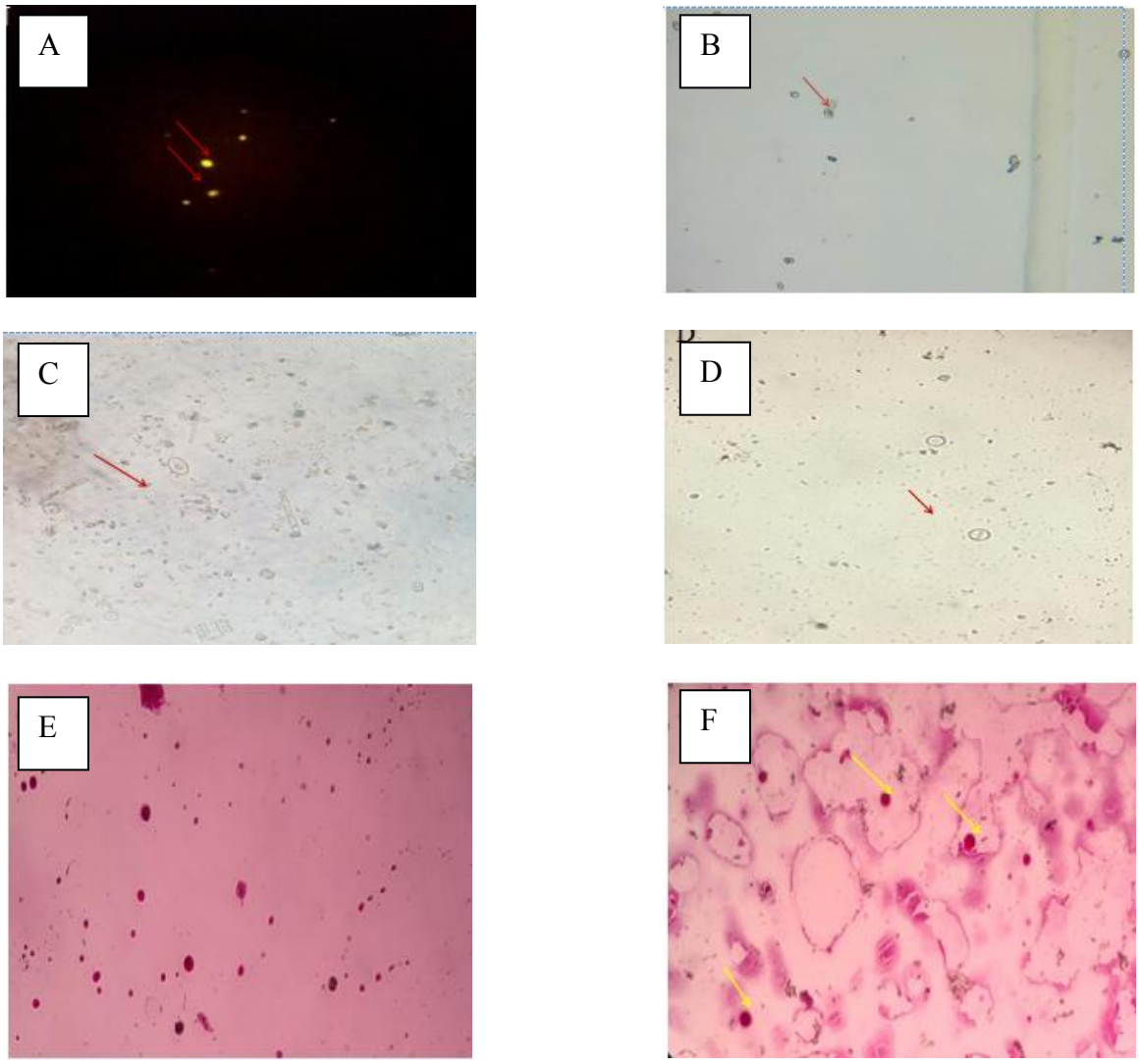
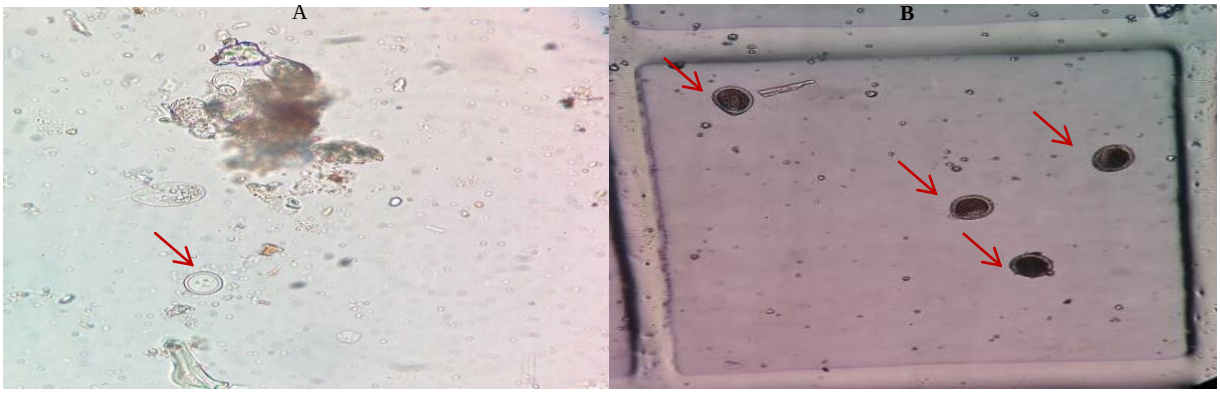


Fig.3 Showing other parasites detected during water examination. A; *Cyclotella algae* (which resembles *Cryptosporidium* oocysts and was differentiated by its inability to take acid fast stain) x1000. B; *Toxocara* found in water samples under S.R. slide (x1000)



Thus, an increase in water contamination is more likely expected in this period of the year (Masina *et al.*, 2019). They also, reported that there was significant relationship between the presence of both parasites in water and seasonal variations.

In our study, one of the main concerns was the high percentage of *C. parvum* (oo) cysts viability (by using DAPI stain) found in potable water samples this may lead to endemic transmission of water-borne parasitic infections.

Cyclotella algae and *Toxocara* were found under microscopy during the examination of surface water samples and documented in Figure 3. *Cyclotella* importance came from being similar to *Giardia* and *Cryptosporidium* fluorescence, after being stained with the IFA reagents as they exhibit a green fluorescence. The presence of fluorescent organisms similar in size and shape to *Giardia* and *Cryptosporidium* organisms increases the likelihood of false-positive results (Rodgers *et al.*, 1995).

Toxocariasis is an infection caused by the ingestion of larvae of the dog roundworm *Toxocaracanis* or the cat roundworm *Toxocaracati*. They may contaminate water and cause human disease that involves the liver, heart, lung, muscle, eye, and brain. It could be transferred through water (Beer *et al.*, 1999).

Our study provided data about the prevalence of *Giardia*sis and cryptosporidiosis in both Tala and Shebin Al-kom water stations as representative of Menoufia Governorate which would provide a database in order to control this public health problem in water supplies.

We found that they are consistently present at high concentrations in raw and potable water samples.

The presence of this high percentage of *C. parvum* viable oocysts in potable water (by using DAPI stain) needs more attention being considered as a risk factor for endemic transmission of water-borne parasitic infections.

The best method for cyst wall lysis should also be documented. Finally, genotyping of examined water samples is recommended to identify the source of infection.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects. However, health education about water contamination and

possible transmitted diseases was done to individuals found in the survey time to minimize risk factors for infections.

Financial assistance

This research was authorized by Menoufia University research unit and supported by a Project fully funded.

Acknowledgment

The authors are grateful to Collaborative Research Center, National Liver Institute. The authors are thankful to Dr/ HeshamAbd-eldayem (NLI dean) for his support and facilitating our work in NLI laboratories.

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How to cite this article:

Dalia Shafey, Mohammed M. Aboamer, Karema Abd Elhady Diab, Heba Mohamed Abdallah, Marwa F. Yousef and Marwa Ahmed Gouda. 2019. Prevalence of *Giardia intestinalis* and *Cryptosporidium parvum* Parasites in Drinking Water in Menoufia Governorate, Egypt. *Int.J.Curr.Microbiol.App.Sci.* 8(12): 2263-2276. doi: https://doi.org/10.20546/ijcmas.2019.812.269