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Municipal Wastewater Treatment Plants as Removal Systems of Virulent Microsporidian Spores.

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ABSTRACT

Municipal wastewater treatment plants play a vital role in reducing the microbial load of sewage. The current study was conducted to investigate the removal of microsporidian spores through municipal wastewater treatment plants (Aslogy and QenayatWWTPs)Sharqeya governorate, Egypt. The detection of microsporidia was carried out by modified trichrome stain (MTS), and molecular technique was used for identification of microsporidia as well as *Enterocytozoonbenieusi* and *Encephalitozoon intestinalis*. The current results showed that the highest removal percentages of microsporidian spores in Qenayat WWTP were recorded in April, May, February and September reaching 100 %, while the removal percentages in other months of the year ranged between 22.2% and 87.6%. In Aslogy WWTP, the removal percentage of microsporidian spores was evenly recorded as 100% in October, December, January, February, April, June, August and September, while it reached 15.8 % in November, 50% in March, 85.7% in May and 74.8% in July. The overall removal percentage of microsporidian spores from Aslogy WWTP was 86.7 %, while it reached 84.2% in Qenayat WWTP.

Keywords: Wastewater treatment plants, Microsporidian spores, MTS, PCR

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INTRODUCTION

Microsporidia are widespread obligate intracellular parasites containing more than 1300 species in 160 genera. Among the 15 species infecting humans, especially *Encephalitozoonintestinalis* and *Enterocytozoonbieneusi*, are the most commonly detected[1-3]. Due to the small size ofmicrosporidian spores, waterborne transmission has not yet been appropriately addressed in epidemiological studies[4].

The demonstration of waterborne microsporidian species known to infect humans proceeding from common waterfowl which have unlimited access to surface waters, has recently been documented by Slodkowicz-Kowalska*et al.*[5]**.** Microsporidia has been confirmed as waterborne protozoon based on its detection in surface water, tertiary sewage effluent, and ground water [6]**.** Although microsporidiosis a concern for AIDS-infected individuals, they are gaining recognition as important infective organisms in immunocompetent individuals as well [7,8].

Environmental sources of human exposure to microsporidia are not known definitively. Fecal-oral transmission is likelybecause many human infections are intestinal and cause severe diarrhea. In disseminating infections of the renal system, microsporidian spores are passed in urine, leading to another possible source of exposure. Also, a strong correlation between soil exposure and microsporidian keratitis in HIV negative patients has been reported [9]**.**

MATERIALS AND METHODS

A total of 96 wastewater samples were collected from two wastewater treatment plants (Aslogy and Qenayat WWTPS).Two wastewater sampling sites (influent and final effluent) were collected from each wastewater treatment plant. Each sampling site was regularly sampled twice per month for one year period from the beginning of October 2012 to the end of September 2013.

Aslogy WWTP produces 10000 cubic meters of treated wastewater per day. The operational system of the plant is composed of: coarse screens, the FOG removal chambers (which contain grease traps, sand, oil traps), primary sedimentation basin, Aeration basin, Secondary sedimentation basin, Effluent Disinfection step in which chlorine is injected to be from 0.5 to 1ppm as a free chlorine final concentration to destroy most of pathogenic microbes.

Qenayat WWTP is composed of: Bar Screen to remove large objects as sticks, cans and debris, which may cause flow obstructions. FOG Removal Chambers, Primary Sedimentation Basinand Trickling Filter: In which an attached-growth, biological process that uses an inert medium to attract microorganisms, Secondary Sedimentation Basin: In which microorganisms and other solids are settled and the final step is disinfection with chlorine: Which is injected to be from 0.5 to 1ppm as a free chlorine final concentration.

Samples (2 liters volume each) were collected in clean polypropylene plastic containers having 10% formalin and sent to the laboratory at the same day of collection.The first portion of each wastewater sample was separately filtered through sterile nitro-cellulose membranes (142 mm diameter and 0.8µm pore size) fitted in stainless steel pressure filter holder that was sterilized before use for each sample. After filtration, the membrane filter was removed from the filter holder and washed three times by the aid of washing solution (1% tween 80) and a stainless-steel bacteriological loop to facilitate the detachment of debris and organisms from the surface of the membrane. The obtained eluent from each sample was collected by centrifugation at 3000rpm for 20 minutes. The supernatant was discarded and the remaining pellet was re-suspended in zinc sulphate floatation solution having 1.2 specific gravity [10]**,** The upper top milliliter was gently aspirated and spread on a clean glass slide and left for air drying and staining with modified trichrome stain according to Weber *et al*. (1992)[11]. The other portion of the same sample was similarly processed as the first portion; except that the finally obtained upper top milliliter (that might contain organisms without debris) was gently aspirated and kept in Eppendorf tube at -20°C until use for PCR.

DNA Extraction:

The preserved part of each sample (the second portion)after processingwas washed with phosphate buffer saline (PBS) and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the pellet was

washed again 2 times as mentioned before. The final pellet was resuspended in 200 µl of PBS. Two hundred microliters of sample suspension were processed by 3 cycles freezing and sawing for extraction of DNA using liquid nitrogen and water bath adjusted at 56°C, then extraction was completed by using the Ez-10 spin column fungal genomic DNA Mini-preps kit according to the manufacture's protocol. After extraction, DNA eluate was stored at −20°C until PCR analysis.

PCR Amplification and Electrophoresis:

PCR was performed using three different diagnostic primer pairs: i) generic microsporidia primer pair (PMP1 and PMP2) used to confirm the presence of microsporidia [12]; ii) species specific primer pair (EBIEF1/EBIER1) for amplification of microsporidian small subunit rRNA (SSU-rRNA) coding regions of *E. bieneusi*[13]**;** and iii) species specific primer pair (SINTF/SINTR) for *E. intestinalis*[14]. Amplification of DNA was performed using Maxima Hot Start Green PCR master mix (Thermo Scientific). A hot-start procedure for microsporidia and *E. bieneusi*was used with an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30s, primer annealing at 60°C for 30s, and extension at 72°C for 30s. A final extension step was performed at 72°C for 10 min [12, 14]. The optimal PCR conditions for the SINTF/SINTR primers were found to be an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 90s. A final extension step was performed at 72°C for 10 min [14]. The PCR products were detected by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

The examination of the influent of Aslogy wastewater treatment plant during one year period from October 2012 to September 2013 revealed that the highest mean count of microsporidian spores was recorded in July (77.5 spores / L), while the lowest mean count of microsporidian spores was recorded in March (one spore / L). On the other hand the microsporidian spore counts in other months ranged between 3.5 and 72 spores/L. The examination of effluent samples from Aslogy WWTP during one year period from October 2012 to September 2013 revealed that the highest mean count of spores was recorded in July (19.5 spores / L), while it reached 0.5 spores / L in each of March and May and 8 spores / L in November. The remaining months of the year had no microsporidian spores. The removal percentage of microsporidian spores in Aslogy WWTP was evenly recorded as 100% in October, December, January, February, April, June, August and September, while it reached 15.8 % in November, 50% in March, 85.7 in May and 74.8 in July (Table 1).

The examination of the influent of the Qenayat wastewater treatment plant during a year period from October 2012 to September 2013 revealed that the highest average of microsporidian spores count was recorded in July (91 spores / L) where the spores was reached zero in October and March, the mean spores count in other months ranged between (4.5 -80.5 spores/ L). The examination of the effluent of Qenayat wastewater treatment plant during one year period from October 2012 to September 2013 revealed that the highest mean spore count was recorded in July (14.5 spores / L), while it reached 2, 3.5, 12 and 10 spores/L in November, December, June and August, respectively. On the other hand, the zero count of microsporidian spores was noted during other months of the year. The highest removal percentages of microsporidian spores in Qenayat WWTP were recorded in April, May, February and September reaching 100 %, while the removal percentages in other months of the year ranged between 22.2 and 87.6%. The overall removal percentage of microsporidian spores from Aslogy WWTP was 86.7 %, while it reached 84.2% in Qenayat WWTP (Table 1).

Table 1: Mean spores count in WWTPs and removal percentage.

interval	Aslogy WWTP			Qenayat WWTP		
	Mean count of spores /1 L		Removal %	Mean count of spores /1 L		Removal %
	Influent	Effluent		Influent	Effluent	
October	4.5	0	100	0	0	…….
November	9.5	8	15.8	11	2	81.8
December	10	0	100	4.5	3.5	22.2

January	9.5	0	100	10	4	60
February	8	0	100	6.5	0	100
March	1	0.5	50	0	0	……
April	7	0	100	8	0	100
May	3.5	0.5	85.7	4	0	100
June	6	0	100	68	12	82.4
July	77.5	19.5	74.8	91	14.5	84.1
August	72	0	100	80.5	10	87.6
September	7.5	0	100	4.5	0	100
Mean	18	2.4	86.7	24	3.8	84.2

Table 2: Paired T-Test and CI: influent of Aslogy WWTP versus effluent of Aslogy WWTP.

Plant type	N	Mean	St Dev	SE Mean
Influent of Aslogy WWTP	12	18	26.67	7.70
Effluent of Aslogy WWTP	12	2.38	5.85	1.69
Difference	12	15.63	23.44	6.77

95% CI for mean difference: (0.73, 30.52)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.31 P-Value = 0.041
P- Value = 0.041 (i.e.< 0.05) this mean that the removal of microsporidian spores was significant through Aslogy WWTP.

Table 3: Paired T-Test and CI: influent of Qenayat WWTP versus effluent of Qenayat WWTP.

Plant type	N	Mean	St Dev	SE Mean
Influent of Qenayat WWTP	12	24.00	34.19	9.87
Effluent of Qenayat WWTP	12	3.83	5.31	1.53
Difference	12	20.17	29.14	8.41

95% CI for mean difference: (1.65, 38.68)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.40, P-Value = 0.035
P- Value = 0.035 (i.e. < 0.05) this mean that the removal of microsporidian spores was significant through Qenayat WWTP.

The microscopic counts of microsporidian spores in positive influents of Aslogy WWTP ranged from 2 to 105 spores/L. with a mean count 22.6 spores/L. Molecular confirmation of the microscopically positive samples for microsporidian spores revealed that only 4 (20%) inlet samples out of the 20 microscopically positive samples for microsporidian spores in Aslogy WWTP were positive by PCR. It was found that the least count of microsporidian spores in PCR positive samples was 50spores/L (i.e. samples containing less than 50 spores gave negative result when tested by conventional PCR technique). All PCR positive-influent samples proved to have 50 – 105 spores/sample. The highest microscopic count of microsporidian spores in effluent samples was 39 spores /sample, while all samples were negative by PCR (Table 4and figure 1).

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Concerning Qenayat WWTP, the microscopic counts of microsporidian spores in positive inlets ranged from 2 to 120 spores/L with a mean count 32 spores/L. Molecular confirmation of the microscopically positive samples for microsporidian spores revealed that only 6 (33%) influent samples out of the 18 microscopically positive samples for microsporidian spores in Qenayat WWTP were positive by PCR (Table 4 and figure 1).

It was found that the least count of microsporidian spores in PCR positive samples was 47spores/L (i.e. samples containing less than 47 spores gave negative result when tested by conventional PCR technique). All PCR positive-influent samples proved to have 47 – 120 spores/sample. The highest microscopic count of microsporidian spores in effluent samples was 29 spores/sample, while all samples were negative by PCR (Table 4).

Table 4: Correlation between microsporidian spore count and PCR-positive samples in examined WWTPs.

Date of sampling		Aslogy WWTP				Qenayat WWTP			
		Influent		Effluent		Influent		Effluent	
		Spores count	PCR	Spores count	PCR	Spores count	PCR	Spores count	PCR
Oct-12	1st sample	9	-ve	0	-ve	0	-ve	0	-ve
	2nd sample	0	-ve	0	-ve	0	-ve	0	-ve
Nov-12	1st sample	9	-ve	16	-ve	10	-ve	4	-ve
	2nd sample	10	-ve	0	-ve	12	-ve	0	-ve
Dec-12	1st sample	20	-ve	0	-ve	9	-ve	7	-ve
	2nd sample	0	-ve	0	-ve	0	-ve	0	-ve
Jan-13	1st sample	9	-ve	0	-ve	4	-ve	2	-ve
	2nd sample	10	-ve	0	-ve	16	-ve	6	-ve
Feb-13	1st sample	8	-ve	0	-ve	8	-ve	0	-ve
	2nd sample	8	-ve	0	-ve	5	-ve	0	-ve
Mar -13	1st sample	2	-ve	1	-ve	0	-ve	0	-ve
	2nd sample	0	-ve	0	-ve	0	-ve	0	-ve
April -13	1st sample	8	-ve	0	-ve	7	-ve	0	-ve
	2nd sample	6	-ve	0	-ve	9	-ve	0	-ve
May -13	1st sample	3	-ve	1	-ve	6	-ve	0	-ve
	2nd sample	4	-ve	0	-ve	2	-ve	0	-ve
Jun-13	1st sample	4	-ve	0	-ve	47	+ve	0	-ve
	2nd sample	8	-ve	0	-ve	89	+ve	24	-ve
July-13	1st sample	50	+ve	0	-ve	62	+ve	0	-ve
	2nd sample	105	+ve	39	-ve	120	+ve	29	-ve
Aug -13	1st sample	90	+ve	0	-ve	110	+ve	20	-ve
	2nd sample	54	+ve	0	-ve	51	+ve	0	-ve
Sep -13	1st sample	15	-ve	0	-ve	0	-ve	0	-ve
	2nd sample	0	-ve	0	-ve	9	-ve	0	-ve
Total positive		20	4	4	0	18	6	7	0

When PCR positive samples for microsporidia were tested by species specific primers, 2 species were detected. The first species was *Enterocytozoonbieneusi* and the second was *Encephalitozoon intestinalis.*

Concerning species identification of microsporidia in PCR positive samples collected from Qenayat WWTP, *Encephalitozoon intestinalis* and*Enterocytozoonbieneusi*were identified in 2 (33.3%) and 6 (100%) of influent samples, respectively (Table 5 and figure1).

Species identification of microsporidia in PCR positive samples collected from Aslogy WWTP revealed presence of *Encephalitozoon intestinalis* and *Enterocytozoonbieneusi* in 1 (25%) and 4 (100%) of influent samples, respectively (Table 5 and figure1).

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Table 5: PCR identification of the detected microsporidian spores.

WWTPs	PCR positive samples	
	No.	Species
Aslogy	4	1*Encephalitozoon intestinalis* 4*Enterocytozoonbieneusi*
Qenayat	6	2*Encephalitozoon intestinalis* 6*Enterocytozoonbieneusi*
Total	10	3*Encephalitozoon intestinalis* 10 *Enterocytozoonbieneusi*

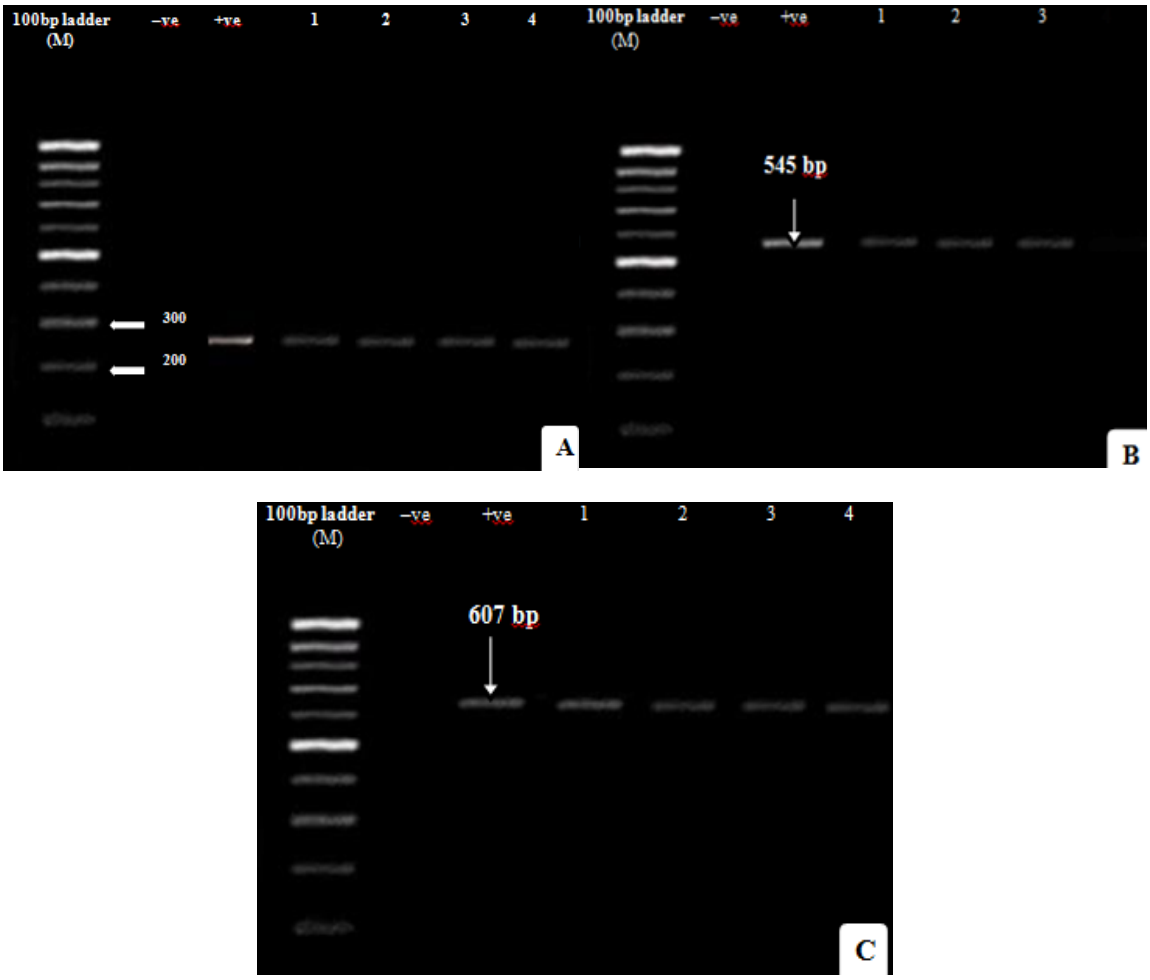

Figure 1: Ethidium bromide stained 2% agrose showing PCR amplified product. M: Marker. -ve: negative control. +ve: positive control. lanes 1-4: positive samples. Plate (A): product of microsporidia. Plate (B): lanes 1-3, product of *Encephalitozoonintestinals.* Plate (C): product of *Enterocytozoonbieneusi*.

DISCUSSION

In the present work, microsporidian spores were microscopically detected in influents and effluents of the 2 examined wastewater treatment plants (Aslogy and QuenayatWWTPs)Sharqeya governorate, Egypt. Microscopic examination of concentrated samples revealed that the prevalence of microsporidian spores reached 51% in the examined WWTPs by staining. Other workers in Spain found that the annual prevalence of microsporidian spores reached 24% of the investigated WWTP by using modified trichrome stain [15]. Also in Spain, different results were obtained as microsporidian spores were detected in 31.3% of the examined WWTP samples by using Weber trichrome stain [16]. Our result was higher than that mentioned by Galvan *et*

al. (2013)[15] and Izquierdoa*et al*. (2011)[16]in Spain and this might be due to difference in climate changes between the two countries.

In Argentina and when permanent trichrome stain was used, it was recorded that the most frequently parasites detected in raw river water were *Microsporidium* spp. (70%) [17].Poma*et al*. (2012)[17] declared that the high percentage of *Microsporidium* spp. in Arsenals river was due to the received raw sewage at different points along the river and also effluents with insufficient treatment from the WWTP[18]**.** In addition, spores of these organisms are potentially resistant to disinfection, similar to other protozoan parasites, like*Cryptosporidium* spp. oocystsand*Giardia* spp. cysts [19]**.**

Concerning the molecular identification of microsporidian spores in the examined WWTPs, it was postulated that spores of *Encephalitozoon intestinalis* were detected in 3 influent samples while spores of *Enterocytozoonbieneusi* were detected in 10 influent samples. All molecularly identified spores were obtained from microscopically positive samples having 47 microsporidian spores or more. All PCR positive samples for microsporidian spores were detected in 2 seasons (spring and summer). The appearance of false-negative results in the present study might be due to a low parasite DNA concentration, and the presence of PCR inhibitors [14]**.** In our opinion the loss of some microsporidian spores during processing of samples and extraction of DNA may lead to inappropriate PCR end results. Other workers in Spain detected microsporidian spores in both influent and effluent wastewater samples by PCR[15]**.** They agreed with the present results in that they detected microsporidian spores in spring and summer seasons. They detected 4 different species (*Enterocytozoonbieneusi*,*Encephalitozoon intestinalis*, *Encephalitozooncuniculi* and *Anncaliiaalgerae*). Also in Spain but in another work, no microsporidian spores were identified in wastewater samples collected during another work[16]**.** In another work conducted in Arizona, USA, the researchers examined only 4 wastewater samples and detected microsporidian spores in 3 of them by PCR [16]**.** Like the present investigation, the predominant species *E. intestinalis* was observed in the effluent wastewater samples.

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