International Journal of Earth, Energy and Environmental Sciences
ISSN: 2517-942X
Vol:11, No:12, 2017

Assessment of Conventional Drinking Water Treatment Plants as Removal Systems of Virulent Microsporidia

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***Abstract*—**Microsporidia comprises various pathogenic species can infect humans by means of water. Moreover, chlorine disinfection of drinking-water has limitations against this protozoan pathogen. A total of 48 water samples were collected from two drinking water treatment plants having two different filtration systems (slow sand filter and rapid sand filter) during one year period. Samples were collected from inlet and outlet of each plant. Samples were separately filtrated through nitrocellulose membrane (142 mm, 0.45 µm), then eluted and centrifuged. The obtained pellet from each sample was subjected to DNA extraction, then, amplification using genus-specific primer for microsporidia. Each microsporidia-PCR positive sample was performed by two species specific primers for *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. The results of the present study showed that the percentage of removal for microsporidia through different treatment processes reached its highest rate in the station using slow sand filters (100%), while the removal by rapid sand filter system was 81.8%. Statistically, the two different drinking water treatment plants (slow and rapid) had significant effect for removal of microsporidia. Molecular identification of microsporidia-PCR positive samples using two different primers for *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* showed the presence of the two pervious species in the inlet water of the two stations, while *Encephalitozoon intestinalis* was detected in the outlet water only. In conclusion, the appearance of virulent microsporidia in treated drinking water may cause potential health threat.

***Keywords*—**Removal, efficacy, microsporidia, drinking water treatment plants, PCR

I. Introduction

WATER treatment technologies have evolved over the past few centuries to protect public health from chemicals and pathogens. Suitable cost effective technologies for developing countries must be considered, as more than a billion of people on the earth have no access to safe potable water that is free from pathogens [1]. Microsporidia are intracellular spore-forming eukaryotic organisms belonging to Phylum Microspora. They are widespread obligate intracellular parasites consisting from 1300–1500 species in about 190 genera [2]-[4]. With recent studies indicating that microsporidia could be classified as fungi; although, they were initially considered to be protozoa. Microsporidia can infect almost all known animal taxa. Ingestion of contaminated food or water with microsporidian spores and person-to-person contact are probably important routes of exposure [5]**.** Among about the 15 species infecting humans, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most commonly detected [2], [3], [6]. Microsporidia has been confirmed as a waterborne parasite based on its detection in surface water [7], drinking water, ground water and tertiary sewage effluent [8]. Because of these findings, microsporidia were listed in the U.S. EPA Contaminant Candidate List (CCL-1, CCL-2) for drinking water [9]**.**

Microsporidiosis can infect immunocompromised as well as immune-competent persons [10]**.** *Encephalitozoon* and *Enterocytozoon* species cause enteric disease but *Encephalitozoon* have a propensity to distribute; practically, *Encephalitozoon* species can infect all organs [11]. Among clinical syndromes associated with disseminated microsporidiosis are keratoconjunctivitis, encephalitis, sinusitis, pneumonia, nephritis, myositis, hepatitis and peritonitis [12].

It was found that 99.9% of *Encephalitozoon intestinalis* spores were inhibited after exposure to free chlorine dose of 2 mg/L for 16 min. *Encephalitozoon cuniculi* and *Encephalitozoon hellem* spores were completely inactivated after exposure to free chlorine dose 2.55 mg/L for 10 min [13].

In Egypt, slow sand filters are used in conventional drinking water treatment plants, but on a small scale, although rapid sand filters are widely used. Recently, numerous slow sand filters systems in drinking water treatment plants were converted to rapid sand filters systems, as it produces a larger quantity of water and requires less space. The available studies on prevalence and removal efficiency of microsporidia in conventional drinking water treatment plants are limited worldwide, and in Egypt are absent. Thus, the main goal of the present study to assess the removal efficiency as well as prevalence of microsporidia in two drinking water treatment plants having two different filtration systems (rapid sand filter and slow sand filter).

II. Materials and Methods

A. Drinking Water Treatment Plants Operational Design

The removal of microsporidia was assessed in two different conventional drinking water treatment plants (DWTPs) located in Fayoum governorate, Egypt. One DWTP was operated by rapid sand filtration system, while the other was operated by a slow sand filtration system. Moreover, slow sand filters required larger land areas compared to rapid sand

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filters, and therefore, they were rarely used in municipal water systems by the 1920s.

The water treatment carried out in the examined DWTPs included coagulation, flocculation, and clarification by sedimentation, filtration, and disinfection by chlorination. A conventional drinking water treatment plant has different treatment steps beginning from the entrance of raw surface water. First step, raw water from the intake is sucked in pipes having coarse metal sieves with pore size 4 cm for prevention of coarse objects from entering the system with the water. In the second step, the sieved raw water is pumped to coagulation and precipitation basins where it is mixed with aluminum sulfate to aid in the flocculation and precipitation of microorganisms and the debris found in raw water. After that, the clear water at the top of the sedimentation basins is collected and passed through sand filters to remove the remaining microorganisms as well as any very small particles that escaped previous filtration. Filtered water is collected in storage tanks where it is injected with a chlorine dose of 2 mg/l for disinfection. The disinfected water (outlet water) is ready to be pumped and distributed to consumers as drinking water [14], [15].

The majority of water filtration plants typically employ rapid sand filtration; therefore, the only filtration that occurs is due to some physico-chemical interactions between the sand and the contaminants and the sand particles hindering large suspended colloidal from passing through the intra-granular space. Its efficiency requires frequent backwashing. Backwashing is an engineering challenge for low technology operating systems. Before engineered filtration using rapid sand filters, often other processes such as coagulation, flocculation, and sedimentation are employed. Slow sand filtration has been municipally used since the 19th century, and continues to be an excellent filtration method. Slow sand filtration is a process involving passage of water through a bed of sand with effective size range 0.15–0.3 mm to a depth of between 0.5 m and 1.5 m at low velocity (generally less than 0.4 m/h) compared with 20 m/h in a rapid granular media filtration, leading to substantial particulate removal by physical and biological mechanisms [1], [16].

Water samples were collected from inlets and outlets of the previously described DWTPs on a monthly basis for one year. Samples (20 liters volume each) were collected in clean polypropylene plastic containers. For each sample, 10 L was concentrated for microscopy and another 10 L for PCR. Each water sample was separately filtered through a sterile nitro-cellulose membrane (142 mm diameter and 0.45 µm pore size) fitted in sterilized stainless steel pressure filter holder. After filtration, the membrane was removed from the filter holder and washed three times with 10 ml washing solution (1% Tween 80) to facilitate the detachment of debris and organisms from the surface of the membrane [17]. The obtained eluents from each sample were collected and centrifuged at 3000 rpm for 10 minutes. The supernatant of outlet water samples was discarded and the remaining few drops were directly used for microscopy and PCR techniques. While for inlet water samples, the supernatant was discarded and the remaining pellet was subjected to flotation technique to collect the spores out of the surrounding debris using zinc sulphate ($ZnSO_4$) solution (specific gravity 1.3), according to Moodley et al. [18]. For each inlet sample, the obtained pellets after purification were processed by microscopic examination and PCR. The obtained pellets for PCR were kept at -20°C until use.

B. Microscopic Detection of Microsporidian Spores

One part of each concentrated sample was spread on clean glass slides and left them for air drying and staining with Weber's chromotrope-based stain for detection of microsporidian spores [19].

C. DNA Extraction

The second preserved portion of each sample was washed three times with phosphate buffer saline (PBS) and each time centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the obtained pellet was resuspended in 200 µl of PBS. DNA was extracted from spores in 200 µl samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol after performing three freeze-thaw cycles, each cycle consisting of 2 min in liquid nitrogen followed by 2 min in boiling water. After extraction, the extracted DNA was stored at −20°C until PCR analysis.

D. PCR Amplification and Electrophoresis

PCR was performed using three different diagnostic primer pairs: i) PMP1 and PMP2 generic primers used to detect microsporidia in all collected water samples [20]. Species identification was performed by PCR using specific primers for *E. bieneusi* and *E. intestinalis* in the PCR-positive samples for microsporidia. ii) For *E. bieneusi,* species specific primer pair (EBIEF1/EBIER1) was used for amplification of microsporidian small subunit rRNA (SSU-rRNA) coding regions [21]**;** and iii) Species specific primer pair (SINTF/SINTR) for amplification *E. intestinalis* [22]. Amplification of DNA was performed using GoTaq® G2 Green Master Mix (Promega, USA). The PCR amplification conditions for microsporidia and *E. bieneusi* were initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 40 s. A final extension step was performed at 72°C for 10 min. The optimal PCR amplification conditions for SINTF/SINTR were as follows: 3 min initial denaturation step at 95^{o}C, 35 cycles of denaturation-annealing-extension at 95^{o}C for 30 s, 58^{o}C for 30 s, and 72^{o}C for 40 s, and a final extension step at 72^{o}C for 10 min. Eight microliters of the PCR product were analyzed on 1.5% agarose gel electrophoresis.

E. Statistical Analysis

The obtained data were analyzed using Paired *t* test and one way ANOVA in Minitab statistical program (Minitab Inc., Pennsylvania, USA). A *P* values less than 0.05 were considered significant.

III. Results

Morphological examination of 24 inlet and outlet water samples from slow sand filtration system drinking water treatment plant (SSFS DWTP) over a one year period revealed the presence of microsporidian spores in 58.3% and 0%, respectively. On the other hand, the prevalence of microsporidian spores was 91.7% and 16.7% in the inlet (n=12) and outlet (n=12) water of a rapid sand filtration system at the drinking water treatment plant (RSFS DWTP), respectively (Fig. 1). The highest count of microsporidian spores in inlet water samples of SSFS DWTP were observed in the summer months. Only five samples out of 12 examined SSFS DWTP inlet samples were proved to be positive for microsporidia by PCR. Contrary to this, microsporidian spores were neither detected by microscopic examination nor by PCR in outlet samples of SSFS DWTP. Concerning species identification, *E. bieneusi* was identified using specific primer in four inlet water samples of SSFS DWTP collected in March, May, June and September. While, *E. intestinalis* was detected by using specific primer only in inlet water sample collected in August (Table I, Fig. 2).

TABLE I
Microsporidian Spores in SSFS DWTP

Interval	Inlet		Outlet	
	Count of spores /10 L	PCR	Count of spores /10 L	PCR
January	0	-ve	0	-ve
February	37	-ve	0	-ve
March	66	*E. bieneusi*	0	-ve
April	0	-ve	0	-ve
May	57	*E. bieneusi*	0	-ve
June	70	*E. bieneusi*	0	-ve
July	40	-ve	0	-ve
August	105	*E. intestinalis*	0	-ve
September	59	*E. bieneusi*	0	-ve
October	0	-ve	0	-ve
November	0	-ve	0	-ve
December	0	-ve	0	-ve

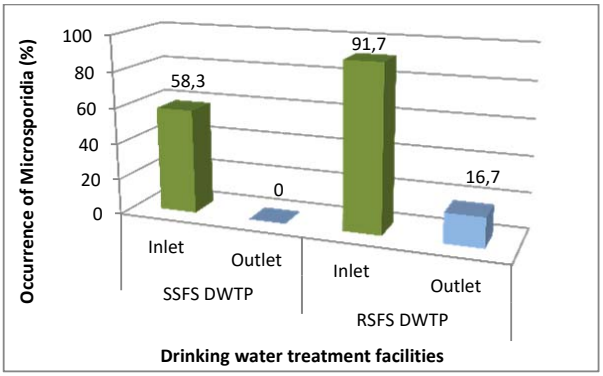

Fig. 1 Prevalence of microsporidia in inlets and outlets of DWTPs

Microsporidian spores were detected in 11 out of 12 examined samples in inlet water of RSFS DWTP using light microscopy. It was noticed that the highest count of microsporidian spores was in August (83 spores/10 L), followed by September (70 spores/10 L) and June (66 spores/10 L). Molecularly, microsporidia were detected in five samples collected from RSFS DWTP inlet. *E. bieneusi* was identified using specific primer in three inlet water samples of RSFS DWTP collected in April, September and October. In addition, *E. intestinalis* was detected only in inlet water samples collected in June and August. All samples collected from the outlet of RSFS DWTP were negative, with the exception of two samples in June and August which were positive for *E. intestinalis* (Table II, Fig 2).

TABLE II
Microsporidian Spores in RSFS DWTP

Interval	Inlet		Outlet	
	Count of spores /10 L	PCR	Count of spores /10 L	PCR
January	0	-ve	0	-ve
February	5	*-ve*	0	-ve
March	10	*-ve*	0	-ve
April	50	*E. bieneusi*	0	-ve
May	30	-ve	0	-ve
June	66	*E. intestinalis*	47	*E. intestinalis*
July	4	-ve	0	-ve
August	83	*E. intestinalis*	57	*E. intestinalis*
September	70	*E. bieneusi*	0	-ve
October	55	*E. bieneusi*	0	-ve
November	5	-ve	0	-ve
December	5	-ve	0	-ve

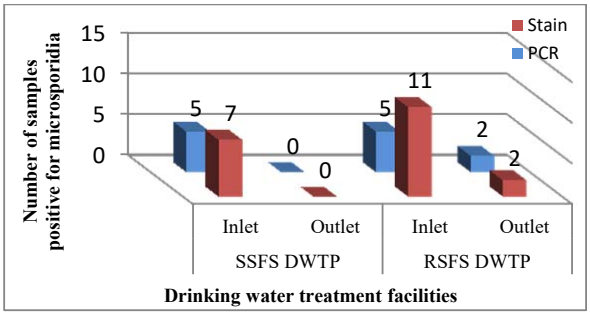

Fig. 2 Comparison between light microscopy and PCR for the detection of Microsporidian spores

Concerning seasonal variations, microsporidian spores reached its highest occurrence (100%) in the inlet water samples of RSFS DWTP collected in three seasons; summer, spring and autumn. On the other hand, the highest and lowest occurrence of microsporidian spores in the inlet samples of SSFS DWTP were recorded in summer (100%) and winter (33.3%), respectively. The prevalence of microsporidia was significantly not affected by the seasons in inlet water samples of RSFS DWTP (p = 0.265) and SSFS DWTP (p = 0.069). The slow sand filtration system drinking water treatment plant was able to remove 100% of microsporidia after complete treatment; while, RSFS DWTP has the ability to remove 81.8% of microsporidia after complete treatment. By conventional statistical criteria, the removal of microsporidian spores after complete treatment in SSFS DWTP was considered to be very statistically significant (p = 0.004). Also, RSFS DWTP was significant (p = 0.006) for the removal of microsporidian spores (Table III, Fig 3).

TABLE III
SEASONAL VARIATION OF MICROSPORIDIA IN INLETS OF THE EXAMINED DWTPS

Season	SSFS DWTP inlet			RSFS DWTP inlet		
	Examined samples	+ve samples	%	Examined samples	+ve samples	%
Winter	3	1	33.3	3	2	66.7
Spring	3	2	66.7	3	3	100
Summer	3	3	100	3	3	100
Autumn	3	2	66.7	3	3	100
Total	12	7	58.3	12	11	91.7

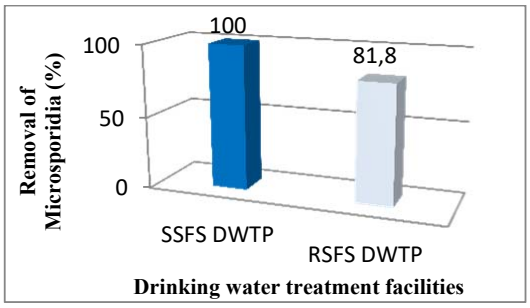

Fig. 3 Removal of microsporidia in the two different DWTPs

IV. DISCUSSION

All water treatment technologies aim to remove turbidity as well as chemical contaminants and pathogens from water sources in expedient manner and at the most affordable detected possible [1]. For eliminating pathogens from drinking water, there are many treatment options. Finding the right solution for a particular supply involves choosing from a range of processes [23]. Slow sand filtration method has been municipally used since the 19th century, and continues to be an excellent filtration method. To date, rare studies were available concerning the removal of microsporidian spores and their species identification in DWTPs. The present study showed that the removal of microsporidia in SSFS DWTP was better than that of RSFS DWTP. Interestingly, the slow sand filtration treatment process has increased in prevalence in the past two decades because its ability to remove chlorine-resistant protozoan pathogens which are responsible for numerous disease outbreaks [23]. In Idaho (USA), a full-scale study for three slow sand filtration drinking water treatment plants showed that no samples were positive for *Giardia* in the treated water from two of the three treatment plants [24]. It was reported that under suitable circumstances, slow sand filtration may be not only the simplest and cheapest but also the most efficient method of drinking water treatment [1]. The vital process in the slow sand filtration is the formation of a biologically active layer (*Schmutzdecke*) in the top 20 mm. A well-established *Schmutzdecke* is responsible for filtration of very small particles, including, parasites, bacteria and viruses [14]. Because the low water productivity of the slow sand filtration, it is therefore suitable for small to medium-sized communities.

The real challenges in drinking water treatments is to control of waterborne transmission of pathogens, because most of the these pathogens produce spores, eggs, oocysts or cysts, which can be difficult to remove in some cases by filtration processes and are extremely resistant to conventionally used water disinfectants [5]. To reduce the risk associated with health problems originated from waterborne diseases, effective removal of pathogens and chemicals should be considered. In the present study, it was found that two outlet water samples from RSFS DWTP were positive for *E. intestinalis*. This result illustrated that the treatment system was not able to remove all microsporidian spores present in the inlet water. To the best of our knowledge, our results are the first report of human-pathogenic microsporidia (*E. intestinalis*) in outlet drinking water samples from Egypt. In other study from Spain, low contamination in DWTPs by

microsporidia (only two cases) occurred in the influent water and no cases in the final treated water [25]. In the present study, it was observed that the microsporidia-positive samples from inlets of DWTPs could be confirmed as *E. bieneusi* and *E. intestinalis* by PCR. Previous PCR studies detected *E. intestinalis* and *E. bieneusi* as human-pathogenic microsporidia in recreational and surface waters [8], [25], [26].

Rapid sand filtration was introduced recently as an alternative technology because slow sand filters occupy large areas of land. Rapid sand filters must be cleaned regularly by 'backwashing, in order to maintain their efficiency. Microsporidian spores are concentrated on the filters during normal operation. Recycling backwash water to the raw water sources can return large numbers of spores to the treatment plant at a time when the plant is potentially vulnerable to breakthrough. This bad practice explains the highest prevalence of microsporidia (91.7%) in the inlet water samples of the examined RSFS DWTP. Also, the insufficient cleaning of sand column of the rapid filter during backwash process lead to escape some microsporidian spores to filtered water and thus cause public health problems. On the other hand, the lowest occurrence of microsporidia (58.3%) in the inlet water samples of the examined SSFS DWTP in the current study due to there being no backwashing for cleaning the slow sand filter, and that the skimmed biological layer of the filter was not discarded in the raw water sources [27].

Although the PCR technique is the most sensitive method for identification of microsporidian species, the results of the present study revealed that morphological detection of microsporidian spores was more productive than molecular detection of microsporidia. The main PCR disadvantage is the appearance of false-negative results, because the presence of PCR inhibitors and a low parasite DNA concentration [22]. An additional reason for a low parasitic microsporidia DNA concentration in water samples is the non-viable empty spores with no DNA, possibly influenced by treatments of DWTPs [25].

V. Conclusions

The presence of virulent microsporidia in treated water samples pointed to the potential health hazards to consumers. The SSFS-DWTP was better than RSFS-DWTP for the removal of microsporidia; moreover both drinking water treatment systems were significant for the removal of microsporidia.

Acknowledgments

The authors are grateful to Mrs. Nora R. Zahglol, Fayoum Drinking Water and Wastewater Holding Company, for her help in collection of water samples. This work was financially supported by National research Centre, Internal project number 11060101.

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