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Reduction of *Cryptosporidium* and *Giardia* by sewage treatment processes

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Abstract. *Cryptosporidium* and *Giardia* are two important pathogenic parasites that have caused many waterborne outbreaks which affected hundreds of thousands of people. Contamination from effluent discharged by sewage treatment plants have been implicated in previous waterborne outbreaks of *Cryptosporidium* and *Giardia*. This study evaluated the reduction of *Cryptosporidium* and *Giardia* (oo)cysts in two sewage treatment plants (STPA and STPB) in Malaysia which employed different treatment processes for a period of a year. Raw sewage influents and treated sewage effluents were concentrated by repeated centrifugation, subjected to sucrose density flotation and concentrated to a minimal volume depending upon the levels of contaminating debris. *Cryptosporidium* oocysts and *Giardia* cysts were enumerated using epifluorescence microscopy. The parasite concentrations in raw sewage were 18-8480 of *Giardia* cysts/litre and 1-80 of *Cryptosporidium* oocysts/litre. In treated sewage, the concentration of parasites ranged from 1-1462 cysts/litre and 20-80 oocysts/litre for *Giardia* and *Cryptosporidium* respectively. Statistical analysis showed that sewage treatment process which employed extended aeration could reduce the concentration of *Cryptosporidium* and *Giardia* (oo)cysts significantly but treatment process which encompasses aerated lagoon could only reduce the concentration of *Giardia* cysts but not *Cryptosporidium* oocysts significantly. This phenomenon is of great concern in areas whereby effluent of sewage treatment plants is discharged into the upstream of rivers that are eventually used for abstraction of drinking water. Therefore, it is important that wastewater treatment authorities rethink the relevance of *Cryptosporidium* and *Giardia* contamination levels in wastewater and watersheds and to develop countermeasures in wastewater treatment plants. Further epidemiological studies on the occurrence and removal of pathogenic organisms from excreta and sewage are also recommended, in order that the public health risks can be defined and the most cost effective sewage treatment options developed.

INTRODUCTION

To date, there have been at least 325 water-associated outbreaks of parasitic protozoan disease documented worldwide (Karanis *et al.*, 2007). *Cryptospordium* and *Giardia*, two notorious pathogenic protozoa have been reported to account for a majority of these outbreaks (132; 40.6% and 165; 50.8%, respectively) (Karanis *et al.*, 2007) affecting hundreds of thousands of individuals (Smith & Grimason, 2003). The evidence of potential role of sewage treatment plant contamination in *Cryptosporidium* outbreak was first highlighted in San Antonio, Texas (D'Antonio *et al.*, 1986). In 1993, contaminated sewage was singled out as the culprit in the largest Milwaukee *Cryptosporidium* waterborne outbreak that sickened 403,000 and killed more than 100 with weakened immune systems. Genetic evaluation data concluded that the outbreak was due to human *Cryptosporidium* type coming from human fecal material and human-associated wastewater (Rose *et al.*, 2002).

Studies on various sewage treatment plants have been carried out throughout the world in developed and developing countries. The occurrence of *Cryptosporidium* and *Giardia* in the sewage treatment plants varies from treatment plants. This variation is due to the differences in the standard of sanitation and water treatment technology in the respective sewage treatment plant (Roach *et al.*, 1993; Wallis *et al.*, 1995; Amahmid *et al.*, 2002; Farias *et al.*, 2002; Scott *et al.*, 2003). Conventional wastewater treatment processes may remove most gastro-intestinal parasites of man, however because *Cryptosporidium* oocysts and *Giardia* cysts are less dense and smaller in size (*Cryptosporidium* = 5 µm; *Giardia* = 8-12 x 7-10 µm) in comparison to helminthic eggs, they may penetrate through wastewater treatment systems more readily. Documented evidence indicated that oocysts and cysts can pass through conventional wastewater treatment processes with reported efficiencies of (oo)cyst removal varying from 79 to 99% for *Cryptosporidium* (Madore *et al.*, 1987) and 40 to 100% for *Giardia* (Sykora *et al.*, 1987) dependent upon the degree of secondary treatment.

In Malaysia, there has not been any reported case of waterborne outbreaks of giardiasis or cryptosporidiosis to date although detection of these two parasites has been reported in human and animal (i.e. cattle, goat, lamb, rodents, birds, dogs and mouse deer) populations, river, well water and raw water of drinking water treatment plants (Ahmad *et al.*, 1997; Lim *et al.*, 1997 & 2005; Lim & Ahmad, 2001, 2004a & 2004b; Lim *et al.*, in press). Abstraction of contaminated river water is evident by the presence of high concentration of (oo)cysts in raw waters of drinking water treatment plants (Lim *et al.*, in press). The possibility of an outbreak happening increases when there is a treatment plant malfunction that enables these parasites to penetrate the treatment processes more easily. Sewage treatment plants have the potential to be a source of contamination to our watershed if the treatment processes employed do not sufficiently treat the effluents before being discharged into nearby river or pond. The efficiency of sewage treatment plants to reducing the concentration of *Cryptosporidium* oocysts and *Giardia* cysts has not been evaluated in Malaysia. The objectives of this study is first to determine and compare the reduction of *Cryptosporidium* and *Giardia* (oo)cysts in two sewage treatment plants (STPs) in Malaysia which employed different treatment processes. Secondly, to determine the level of *Cryptosporidium* and *Giardia* (oo)cysts contamination into the receiving waters.

MATERIALS AND METHODS

Sewage treatment plants (STPs)

The two sewage treatment plants (STPs) studied are designated as Plant A and Plant B. The characteristics of the sewage treatment plants are described in Table 1.

Sample collection, isolation and detection of oocysts

Ten litres of sewage water was sampled monthly for a period of a year from the raw sewage influent and treated sewage effluent of treatment plant A and B. The influents and effluents were concentrated by repeated centrifugation at 1500 x g for 10 min in 50 mL conical bottomed centrifuge tubes before being brought down to 10 mL. Samples were slowly under layered with 10 mL of cold sucrose solution (a solution with a specific gravity of 1.18) and centrifuged (1000 x g, 5 min). The entire supernatant including the interface was recovered without disturbing the pellet and decanted gently into a clean centrifuged tube. The residual sucrose was removed by washing three times in pH 7.2 phosphate buffered saline (PBS) (Ovoid, Hampshire, UK). The final concentrated sample was reduced to a final volume of 1 to 5 mL, depending on how turbid the samples were.

Fifty µl aliquots of each sample concentrate were placed onto each of a single Teflon®-coated microscope slide and air dried (room temperature = 24 ± 1°C). Samples were methanol-fixed and then

Table 1. Main features of the treatment plants processes

Sewage treatment plant	STPA	STPB
Loading / Population equivalent (PE)	50,180	124,035
Treatment type	Extended Aeration (EA)	Aerated Lagoon (AL)
Primary treatment	Screening and grit separation	Screening and grit separation
Secondary treatment	Oxidation with O_2 (EA introduces air in the form of fine bubbles through submerged diffusers. Fine bubbles promote higher oxygen transfer efficiency) and sedimentation	Oxidation with O_2 (AL uses surface aerators to provide air) and sedimentation
Disinfection	None	None
Retention time	8 to 24 hours	1 to 2.5 days

overlaid with commercially available fluorescein isothiocyanate (FITC)-labelled anti-*Giardia* cysts monoclonal antibody (mAb) and (FITC)-labelled anti-*Cryptosporidium* oocysts mAb (Crypto/Giardia Cel IFA, Cellabs, Australia). Slides were incubated in a humidity chamber for 30 ± 5 min at 37°C. Excess antibody was removed by rinsing the slides twice with 50 µL of PBS dropped onto each well. The presence of sporozoite nuclei was highlighted by staining them with aliquots of 2 mg/ml DAPI (4',6-diamidino-2-phenylindole) (Sigma Chemical, Missouri, USA) solution for 2 minutes and later rinsed with distilled water. Twenty µL of mounting medium (PBS:glycerol, 1:1 v/v) was mounted onto each well and cover slips were applied to the slides which were then examined under a x400 epi-fluorescence microscope (Olympus Microscope Model BX52, Japan) equipped with wide band excitation colour separation filter (exciter 460-490 nm; barrier 550 nm) to detect FITC stain and a wide band UV filter (exciter 330-335 nm, barrier 420 nm) for DAPI stain. Internal morphology of (oo)cysts was observed by using Normaski DIC microscopy at x1000 magnification. Positive control slides containing cysts and oocysts and negative control slides were included in the analysis.

Statistical analysis

The normality of the data was tested using the one-sample Kolmogorov-Smirnov Test. Based on the normality results, the reduction in the concentrations of *Giardia* in the effluents from the influents was tested using paired T-test whereas the reduction in the concentrations of *Cryptosporidium* in the effluents from the influents was tested using Wilcoxon Signed Ranks Test.

RESULTS

Overview

A total of 48 samples were collected from the influent and effluent points of STPs A and B for a period of a year. Generally, *Giardia* cysts and *Cryptosporidium* oocysts were detected both in raw and treated sewage with *Giardia* always outnumbering *Cryptosporidium*. *Giardia* cysts were present in 100% raw and treated sewage from both STPs. However, 50% of raw and 25% of treated sewage were positive for *Cryptosporidium* oocysts. The parasite concentrations in raw sewage were 18-8480 of *Giardia* cysts/litre and 1-80 of *Cryptosporidium* oocysts/litre. In treated sewage, the concentration of parasites ranged from 1-1462 cysts/litre and 20-80 oocysts/litre for *Giardia* and *Cryptosporidium* respectively.

Sewage treatment plant A (STPA)

A total of 24 samples (12 raw sewage influents, 12 treated sewage effluents) were collected monthly for a period of a year from Sewage Treatment Plant A (STPA). *Giardia* cysts were detected more frequently and at higher concentrations compared to *Cryptosporidium* oocysts both in the raw influent and treated effluent. *Giardia* cysts (range 18 to 5240 cysts/litre) were consistently detected in 12 (100%) however *Cryptosporidium* oocysts (1 to 80 oocysts/litre) in 8 (66.7%) raw sewage influents examined. Treated sewage effluents of treatment plant A contained *Giardia* cysts (1 to 500 cysts/litre) in 12 (100%) and *Cryptosporidium* oocysts (20 to 40 oocysts/litre) in 3 (25%) effluents examined. The average rate of reduction was estimated to be higher for *Giardia* (96%) than *Cryptosporidium* (73%) (Figure 1). According to the paired t-test, there was a significant difference ($p<0.05$) between the concentrations of *Giardia* in the raw influents and treated effluents of STPA indicating significant reduction for *Giardia* cysts. Significant ($p<0.05$) reduction for *Cryptosporidium* oocysts was also shown by Wilcoxon Signed Ranks Test between the concentrations of *Cryptosporidium* in the influents and effluents of STPA. This data showed that the sewage treatment processes in STPA was sufficient in significantly reducing both the concentration of *Giardia* and *Cryptosporidium* (oo)cysts.

Sewage treatment plant B (STPB)

Twenty four samples (12 raw sewage influents, 12 treated sewage effluents) were collected monthly for a period of a year from Sewage Treatment Plant B (STPB). *Giardia* cysts were present in all raw and treated sewage, however, only 25% (3 samples) of raw and 25% (3 samples) of treated sewage were positive for *Cryptosporidium* oocysts. The concentrations of *Giardia* outnumbered *Cryptosporidium* both in raw (*Giardia*: 55 to 8480 cysts/litre; *Cryptosporidium*: 40 to 80 oocysts/litre) and treated samples (*Giardia*: 28 to 1462 cysts/litre; *Crypto-*

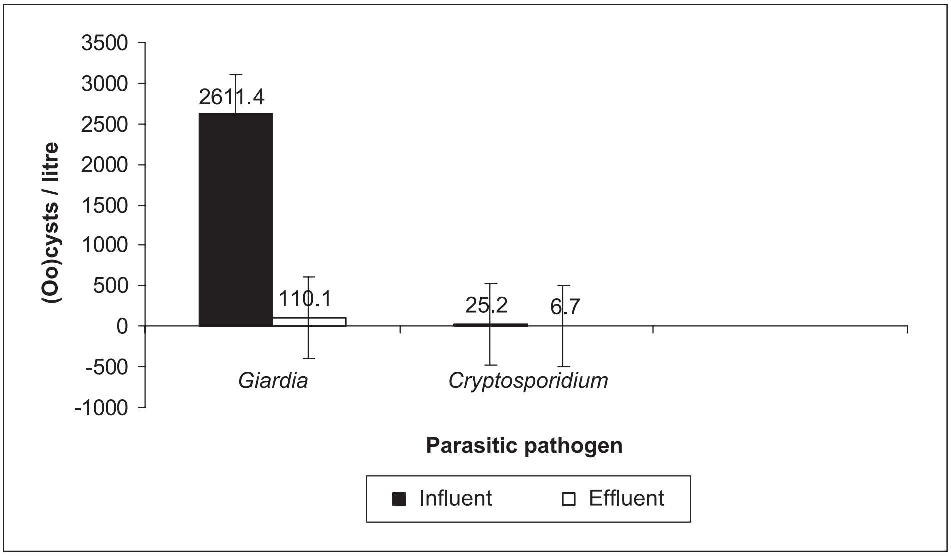

Figure 1. Mean (oo)cysts/litre of *Cryptosporidium* and *Giardia* in the influent and effluent samples of Sewage Treatment Plant A over a period of 12 months.

sporidium: 20 to 80 oocysts/litre). The average rate of reduction was estimated to be much higher for *Giardia* (92%) than *Cryptosporidium* (33%) (Figure 2). Statistical analysis according to the paired t-test indicated that there was a significant ($p<0.05$) reduction of *Giardia* cysts concentrations after treatment of sewage at STPB. However, according to the Wilcoxon Signed Ranks Test, there was no significant ($p>0.05$) reduction of *Cryptosporidium* oocysts by the processes at STPB. This showed that the sewage treatment processes at STPB was sufficient in significantly reducing the numbers of *Giardia* cysts but not *Cryptosporidium* oocysts.

DISCUSSION

There has been quite a substantial amount of work on *Cryptosporidium* and *Giardia* in Malaysian potable and surface waters. However, relatively little information is available on the occurrence of these pathogens in waste-waters. This study attempts to redress this imbalance and reports on the occurrence of *Cryptosporidium* and *Giardia* in raw and treated sewage.

Both parasites were detected frequently (100% of sewage samples were *Giardia* positive and 35.4% were *Cryptosporidium* positive) and at maximum concentrations of >8,000 parasites/liter in the raw sewage samples. *Giardia* persistently outnumbered *Cryptosporidium* in both raw and treated sewage samples. Raw sewage contained 18 to 8480 *Giardia* cysts/litre and 1 to 80 *Cryptosporidium* oocysts/litre. Treated sewage contained 1 to 1462 *Giardia* cysts/litre and 20 to 80 *Cryptosporidium* oocysts/litre. Other studies conducted in Sweden, Norway and Canada also reported of constant detection of *Giardia* in sewage (Ottoson *et al.*, 2006; Robertson *et al.*, 2006) and a rather erratic occurrence of *Cryptosporidium* (Payment *et al.*, 2001). In contrast to these findings, previous investigators using large volume filtration techniques have reported significantly greater number of *Cryptosporidium* oocysts compared to *Giardia* cysts (DeLeon *et al.*, 1988).

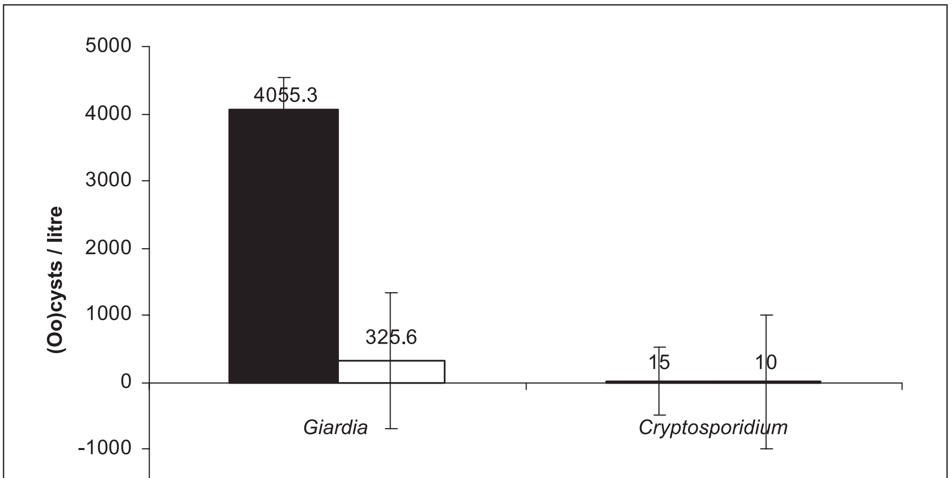

Figure 2. Mean (oo)cysts/litre of *Cryptosporidium* and *Giardia* in the influent and effluent samples of Sewage Treatment Plant B over a period of 12 months.

The higher concentrations of *Giardia* cysts in raw sewage could suggest that giardiasis is more widespread, and/or occurred with greater infection intensity than cryptosporidiosis. This is supported by epidemiological data for Malaysia that indicated giardiasis was more common among communities and had higher prevalence rates (range: 0.21-25%; average >10%) (Bisseru & Aziz, 1970; Dunn, 1972; Dissanaike *et al.*, 1977; Nawalinski & Roundy, 1978; Sinniah *et al.*, 1978, 1988; Hamimah *et al.*, 1982; Sinniah, 1984; Che Ghani *et al.*, 1987; Sinniah & Rajeswari, 1988; Lai, 1992; Che Ghani, 1993; Kamel *et al.*, 1994a; Rajeswari *et al.*, 1994; Shekhar *et al.*, 1996; Lim *et al.*, 1997; Rahmah *et al.*, 1997; Norhayati *et al.*, 1998; Menon *et al.*, 1999) compared to cryptosporidiosis (range: 0.9-23%; average <10%) (Che Ghani *et al.*, 1984; Mendez *et al.*, 1988; Mat Ludin *et al.*, 1991; Lai, 1992; Ng & Shekhar, 1993; Kamel *et al.*, 1994a, 1994b; Lim *et al.*, 1997, 2005; Menon *et al.*, 1999, 2001). This variation was also noted in other countries (i.e. Norway) (Robertson *et al.*, 2006).

The amount of parasites in the influent of a sewage treatment plant depends on the size of the community served as well as the rate of infection within the population (Smith & Rose, 1990). STPs serving higher person equivalents were more likely to be positive and have higher parasite concentrations (Robertson *et al.*, 2006). This trend was also observed in this study. The parasites (both *Giardia* and *Cryptosporidium*) concentrations in raw sewage at STPA (1-5240 (oo)cysts/litre) which has a loading capacity of fifty thousand population equivalent were lower than the parasites concentration in raw sewage at STPB (40-8480 (oo)cysts/litre) which had twice of STPA's loading capacity.

In the study in Norway, parasite concentrations in sewage were also used to estimate the proportion of contributing populations that could be clinically infected. For *Cryptosporidium*, the highest estimates were up to 5 per 100,000 individuals for two populations in eastern Norway. For *Giardia*, the highest estimate was 40 infected per 100,000 persons (approximately five times the usual national annual average) contributing to an STW in western Norway (Robertson *et al.*, 2006). Another advantage of examining parasites in sewage was that it gives a more representative indication of endemic infection than epidemiological data, as it takes into account all of the asymptomatic carriers and misdiagnosed cases that ordinarily escape detection. (Jakubowski *et al.*, 1991). Giardiasis and cryptosporidiosis are often grossly underreported in Malaysia because these two diseases are not notifiable diseases in Malaysia.

The presence of high concentrations of *Giardia* cysts (1-1462 cysts/litre) and *Cryptosporidium* oocysts (20-80 oocysts/litre) in the effluent is of public health concern because the effluent is directly discharged into the river causing environmental contamination with these parasites. It poses health risk when the river water is being used by communities living downstream of river for household consumption and usage.

The type of treatment processes plays an important role in determining the reduction of parasites such as *Giardia* and *Cryptosporidium* as indicated in this study. The removal rates at STPA which employs extended aeration system (*Giardia* 96% and *Cryptosporidium* 73%) were better than STPB which employs aerated lagoon system (*Giardia* 92% and *Cryptosporidium* 33%). Statistical analysis showed that the processes at STPA significantly reduced the numbers of *Giardia* and *Cryptosporidium* (oo)cysts, however processes at STPB only significantly reduced *Giardia* cysts but not *Cryptosporidium* oocysts. Both treatment plants have the same primary treatment but differ in their secondary treatment. In STPA, the secondary treatment consists of oxidation with O_2 by introducing air in the form of fine bubbles through submerged diffusers. Fine bubbles promote higher oxygen transfer efficiency whereas in STPB, the oxidation with O_2 uses surface aerators to provide air.

This study showed that the average removal efficiencies at these two sewage treatment plants which have both primary and secondary treatment processes are approximately 50% for *Cryptosporidium* and > 90% for *Giardia*. Interpretation from various studies reported that the removal of *Giardia* cysts by sewage treatment can be estimated to be 60-90% in STW incorporating a primary and secondary process but may vary from under 10% to over 90% for *Cryptosporidium* oocysts (Robertson *et al.*, 2000, 2006). Factors which affect parasite removal will include properties of the parasites themselves, the sewage treatments and the interactions between parasite and treatment (Robertson *et al.*, 2000). Studies on STWs with minimal treatment had negligible removal of both parasites. In Malaysia, more than 50% of sewage treatment plants only have primary treatment process. The risk of contamination of water courses by *Cryptosporidium* and *Giardia* is considerable when the effluent is discharged into rivers and lakes.

Results indicated that sewage treatment process which employed extended aeration could reduce the concentration of *Cryptosporidium* and *Giardia* (oo)cysts significantly but aerated lagoon could only reduce the concentration of *Giardia* cysts but not *Cryptosporidium* oocysts significantly. Sewage treatment plant utilizing better technology gave a better though not total reduction of these parasites. Levels of *Giardia* and *Cryptosporidium* contamination into receiving waters were high as indicated by the high concentrations of parasites in the effluent (1 to 1462 *Giardia* cysts/litre and 20 to 80 *Cryptosporidium* oocysts/litre). This phenomenon is of great concern because effluent is directly discharged into rivers, thus, risk of contamination of water courses by *Cryptosporidium* and *Giardia* is considerable. It poses health risk when the river water is being used by communities living downstream of river for household consumption and usage. Therefore, it is important that wastewater treatment authorities rethink the relevance of *Cryptosporidium* and *Giardia* contamination levels in wastewater and watersheds and to develop countermeasures in wastewater treatment plants. Further epidemiological studies on the occurrence and removal of pathogenic organisms from excreta and sewage are also recommended, in order that the public health risks can be defined and the most cost effective sewage treatment options developed.

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