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EVALUATION OF TWO CONCENTRATION METHODS FOR DETECTING *GIARDIA* AND *CRYPTOSPORIDIUM* IN WATER

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Abstract—The cartridge filtration method and membrane filtration method based on the fluorescent antibody procedure were evaluated for their recovery efficiencies and detection limits of *Giardia* and *Cryptosporidium*. We assessed the performances of the two concentration methods for water samples collected from Taiwan water treatment plants. The membrane filtration method was characterized by higher recovery rate and detection limit comparing with the cartridge filtration method. The occurrences of both parasites, and the relationships of parasite concentrations with indicator microorganisms show inconsistency between the two methods. It was discovered that water turbidity reduced the recovery efficiencies, and raised the detection limits for both parasites regardless of the method used.

Key words—*Giardia, Cryptosporidium,* water treatment, ICR

INTRODUCTION

Protozoan Parasites, *Giardia* and *Cryptosporidium*, have been recognized as common pathogenic protozoa of the gastrointestinal tract (Cook, 1995). Many outbreaks of giardiasis and cryptosporidiosis have been reported in the last few decades (Frost *et al*., 1996; SoloGabriele and Neumeister, 1996; Kramer *et al*., 1996). Water is perhaps the major route for massive outbreaks of pathogen infection, as a result of contamination of either raw or treated water (Teunis *et al*., 1997; Eisenberg *et al*., 1998; Perz *et al*., 1998). The occurrence of *Giardia* and *Cryptosporidium* in drinking water has aroused attention on detection of the protozoa at levels concerning human health.

Methods available for the detection of *Giardia* and *Cryptosporidium* in water sample generally contains three stages: sample concentration and elution; separation of cysts and oocysts from other debris; staining and identification of the protozoan parasites. Information Collection Requirement Rule (ICR) proposed a standard method for detecting *Giardia* and *Cryptosporidium* in water samples by the fluorescent antibody procedure (USEPA, 1995), which, however, has been heavily scrutinized by many researchers, such as Clancy *et al*. (1994) and LeChevallier *et al*. (1995). Investigation shows that the cartridge filters concentration procedure and Percoll-sucrose flotation technique are the primary causes for the poor recovery performances. Method 1622 (USEPA, 1997,1999a) and Method 1623 (USEPA, 1999b), with an improved concentration procedure and adoption of immunomagnetic separation (IMS), are expected to have higher recovery and lower detection limit. However, most water utilities still rely on the ICR method and its forerunner—ASTM P229 or modification regardless of its high variability and low sensitivity. It is because that Method 1622 and 1623 is only applicable at low turbidity as well as its high cost of equipment and materials (Clancy and Hansen, 1999; Atherholt and Korn, 1999).

The aim of this study was to evaluate the two concentration methods, the cartridge filtration proposed in ICR protozoan method and the membrane filtration recommended by Method 1622 draft, based on their sensitivities, recovery efficiencies and detection limits on cysts and oocysts. The correlation between the densities of protozoan parasite and some indicator microorganisms were determined, and the effect of turbidity on these two methods was also evaluated.

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MATERIALS AND METHODS

Preparation of protozoa-contained sample and collection of water sample

The cysts and oocysts were obtained from Waterborne, Inc. (Louisiana, USA) and the Pleasant Hill Farms (Idaho, USA), respectively, and were diluted to desired concentrations by 0.1% PBS as the stock solution. The numbers of cysts and oocysts in the stock solution were counted before each seeded experiment using the immunofluorescence assay technique. To do this, samples were mixed thoroughly and pipetted directly from the stock preparation vial and then onto the glass slides (Dynal® Spot-On), stained with fluorescent-labelled antibodies (HydrofluorTM Combo *Giardia*/*Cryptosporidium*; Ensys, Inc., NC, USA), and counted. Water samples for seeding were taken from the Hsin-Chu water treatment plant, located in northern Taiwan. The waters were seeded with 10^3–10^4/l of cysts and oocysts to determine the recovery efficiencies. To detect the occurrence of parasites in Taiwan, 20 raw water samples and 20 treated water samples from five public water treatment plants, as well as 12 raw water samples from six simple water facilities were collected.

Concentration, elution and clarification technique

Cartridge filtration apparatus include a 25.4 cm long and 1.0 μm nominal-pore-size polypropylene yarn-wound cartridge filter (M39R10A; Commercial Filters Parker Hannifin Corp., IN, USA) with LT-10 filter holder. Membrane filtration apparatus include polycarbonate membrane (142 mm diameter, pore size 3 μm; Catalog No. 16565; Osmonics Inc., USA) with 142 mm stainless-steel filter holder (Millipore Corp., MA, USA).

Aliquots of seeded samples and unseeded samples were filtered through cartridge filters. The filters were removed from the cartridge, cut off from the supporting core, and divided into three parts. The filter fibers were eluted with 0.7–1.2 l eluting fluid (phosphate-buffered saline, 1% Tween 80, 1% SDS) in a mechanical stomacher, and then the eluate was centrifuged at 1050 g for 10 min in a 50 ml centrifuge tube in swinging-bucket rotor. The volume of the packed pellet was recorded. After the supernatant was aspirated, the pellet was resuspended in an equal volume of 10% formalin and the eluting fluid was added to a total volume of 20 ml in the same centrifuge tube. The mixture was under-laid with 30 ml of Percoll-sucrose gradient (sp. gravity = 1.10) followed by centrifuging. The top 20 ml and 5 ml below the interface were collected, diluted with eluting fluid to 50 ml, and centrifuged. The upper-layer liquid was then aspirated till only 5 ml of concentrate was left.

The same water samples as those used in the cartridge filtration method were also passed through 142-mm-diameter polycarbonate membranes in a stainless-steel filter holder. Protozoan parasites were removed from the membrane by scraping, followed by washing with the eluting fluid (phosphate-buffered saline, 1% Tween 80, 1% SDS). The eluate was transferred into a 50 ml conical centrifuge tube, and centrifuged for 15 min at 1050 g for 10 min. After aspirated the top 45 ml solution, the remaining pellet was preserved with equal volume of 10% formalin. Clarification was done by Percoll-sucrose gradient, as described previously.

Staining and examination

The cysts and oocysts in the water samples were stained and examined following the procedures described in the ICR protozoan method for detecting *Giardia* cysts and *Cryptosporidium* oocysts in Water by a fluorescent antibody procedure (USEPA, 1995). To do this, the re-suspended sediment samples were applied to each 25 mm diameter cellulose–acetate membrane, stained with fluorescent-labelled antibodies (Hydrofluor™-Combo *Giardia*/*Cryptosporidium*; Ensys, Inc., NC, USA), and examined with epifluorescent microscope at 200×, 400× or 1000× magnification (Olympus, Japan). The antibody, Hydrofluor™-Combo *Giardia*/*Cryptosporidium*, can react with all species of *Giardia* as well as *Cryptosporidium parvum*, *Cryptosporidium muris*, and *Cryptosporidium meleagridis*. Cysts and oocysts were identified using following parameters: size, shape, surface feature, and staining reaction. The candidates exhibiting right size and shape were further identified by their internal morphological features with epifluorescent microscopy under a bright field.

Data analysis and calculation

To investigate the recovery efficiency of the cartridge filtration method and membrane filtration method, we visually counted the numbers of cysts and oocysts of the seeded water samples before filtration and after clarification through epifluorescent microscope. The detection limit of each water sample was calculated as described in equation (1) (USEPA, 1995).

$$X = \frac{100}{V \times F \times R} \tag{1}$$

where X is the detection limit of cysts/oocysts per 100 l of the water sample, V is the water volume, F is the fraction of the initial eluate packed-pellet volume subjected to clarification, R is the percentage of floated sediment examined. The numbers of cysts and oocysts observed under the microscope were recorded, multiplied by the detection limit and divided by the recovery efficiency to obtain the final counts. When no protozoa was observed under the epifluorescent microscope, we expressed the protozoa concentration in water samples as less than (<) their detection limits. In the statistical evaluation, data under the detection limits were treated as zero. Water turbidity was measured using a ratio turbidimeter (HACH, Co., USA). Heterotrophic bacteria were measured by the spread method (APHA, 1995), while other indicator microorganisms (total coliforms, fecal coliforms, *E.* coli, and Enterococcus) were measured by membrane filtration procedures described by the standard method for the examination of water and wastewater (Methods 9222 B in APHA, 1995). Spearman R correlation coefficients were calculated between the concentrations of cysts/oocysts and the indicator microorganisms, and between the detection results from cartridge filtration method and membrane filtration method, using the STATISTICA software (StatSoft, Inc., USA).

RESULTS AND DISCUSSION

Recovery efficiency of two concentration methods in seeded water

The mean recovery efficiencies of protozoan parasites concentrated by two methods are shown in Fig. 1. The recovery efficiencies of concentration by cartridge filter for 40 l of raw and treated water were $28.4 \pm 11.0\%$ ($n = 4$) and $30.0 \pm 11.7\%$ ($n = 8$) for cysts, $9.3 \pm 3.3\%$ ($n = 4$) and $9.8 \pm 4.5\%$ ($n = 8$) for oocysts, respectively. Those by membrane filter for 20 l of treated water were $40.4 \pm 9.7\%$ ($n = 5$) for cysts and $17.5 \pm 3.2\%$ ($n = 5$) for oocysts. For membrane filter, less than 2 l of raw water were concentrated, and their mean recovery efficiencies are $38.3 \pm 18.5\%$ ($n = 4$) for cysts and $16.0 \pm 1.7\%$ ($n = 3$) for oocysts, respectively. LeChevallier *et al.* (1995) and Clancy *et al.* (1994) have concluded the

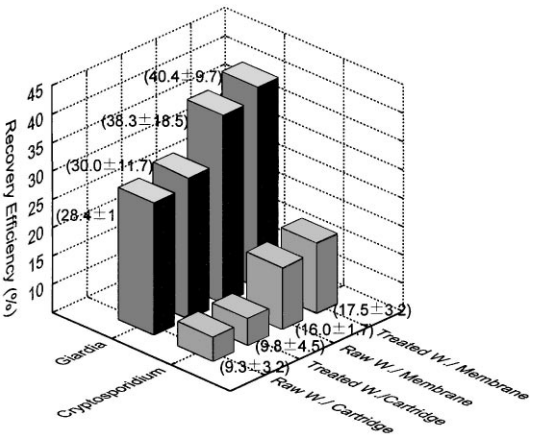

Fig. 1. The mean recovery efficiencies of *Giardia* and *Cryptosporidium* detected by two concentration methods from seeded water samples.

presence of unidentified variation in evaluating recovery efficiencies, and the values from one lab to another can vary by logarithmic factors of 0.31. LeChevallier *et al.* (1995) also suggested that careful handling in concentration and clarification may improve the recovery efficiency.

Our analysis showed higher protozoa recovery for treated water samples than for raw water samples, although not statistically different, which was similar to that reported in the literature (LeChevallier and Norton, 1995). Our results also showed that membrane filtration method had higher recovery efficiency than cartridge filtration method regardless of the types of water samples. Similar results were observed by Nieminski *et al.* (1995), and Shepherd and Wyn-Jones (1995). *Cryptosporidium* oocysts showed lower recovery than *Giardia* cysts from both concentration methods. The results are in agreement with the findings from related studies (Falk *et al.*, 1998; Nieminski *et al.*, 1995; LeChevallier *et al.*, 1995) that oocysts lodge in the deeper area of the cartridge/membrane filters due to their small size, which makes them difficult to remove during the elution procedure.

Concentrations of Protozoa and their detection limits in water samples

The five large water treatment plants in this study use the traditional treatment processes, which including coagulation, flocculation, sedimentation, filtration and disinfection. The average residual chlorine concentration in treated water determined as 0.743 ± 0.169 mg/l ($n = 10$). The water from six simple water facilities is treated by simple filtration procedure, because these systems located in the secluded area generally have fewer technical and financial resources to cope with new requirements.

Water samples from water treatment plants and simple water facilities in this study were filtrated through both types of filters. The mean detection limits of parasites for these two concentration methods are shown in Fig. 2. Among these three types of water samples, treated water displayed the lowest mean detection limit (5.2 ± 2.6 organisms/100 l for cartridge filtration method and 20.5 ± 16.0 organisms/100 l for membrane filtration method), while raw water showed the highest mean detection limit (14.4 ± 4.7 organisms/100 l for cartridge filtration method and 259.3 ± 289.6 organisms/100 l for membrane filtration method). The detection limits of cartridge filtration method for all samples were quite consistent while membrane filtration method exhibit much greater variation in detection limits.

Due to high turbidity of raw waters, only a small volume of sample could be processed through the membrane filter. This resulted in a significantly high mean detection limit, 259.3 ± 289.6/100 l, which was 16 times higher than that of treated water and 10.4 times higher than the simple facility water. Such a small sampling volume also caused the error in calculating cysts or oocysts in water samples when converting to the number of parasites per 100 l due to the fact that parasites are not uniformly distributed in water samples (Atherholt and Korn, 1999). The ICR requires that large water utilities monitor the treated water whenever the *Cryptosporidium* concentrations in raw water exceed 100 oocysts/100 l (LeChevallier and Norton, 1995). The membrane filtration cannot replace the cartridge filtration, because any number of cyst or oocyst detected on the slide would have generated a concentration that exceeds the ICR regulation.

The concentrations of *Giardia* and *Cryptosporidium* in 52 water samples were listed in Table 1. Although the water samples used in two concentration methods were collected and concentrated simultaneously, the occurrence of parasites detected by two methods varied significantly. Due to the differences in detection limits, errors in the measurement procedure, and variation in sampling, the percentage samples of conflicting result were 23 and 27% for

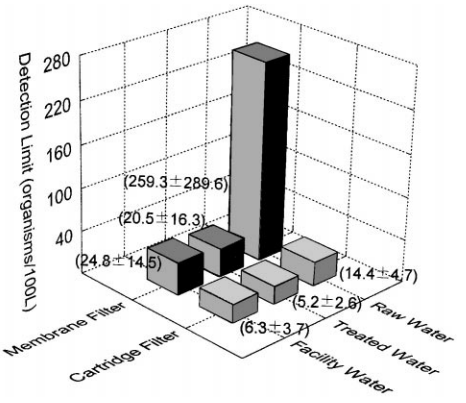

Fig. 2. The mean parasite detection limits of two concentration methods from different types of water samples.

Table 1. The concentration of *Giardia* and *Cryptosporidium* in 52 water samples in Taiwan water supplies

Sampling site	Raw water/cartridge filter		Raw water/membrane filter		Treated water/cartridge filter		Treated water/membrane filter	
	Giardia (cysts/100 l)	*Cryptosporidium* (oocysts/100 l)	*Giardia* (cysts/100 l)	*Cryptosporidium* (oocysts/100 l)	*Giardia* (cysts/100 l)	*Cryptosporidium* (oocysts/100 l)	*Giardia* (cysts/100 l)	*Cryptosporidium* (oocysts/100 l)
B.D.	540	117	4929	<5887	123	189	<130	<300
B.D.	3175	921	3190	1524	92	157	687	<113
P.H.	119	<180	350	712	63	292	<108	<250
P.H.	554	613	11,027	2441	103	<38	49	<29
H.C.	<29	2097	425	2033	<17	<52	41	<95
H.C.	583	888	3268	7813	143	183	41	143
F.Y.	28	<84	<54	130	<22	<67	<31	<71
F.Y.	286	871	349	3333	31	32	12	86
C.C.	41	<125	<622	<2437	<9	<27	<61	<141
C.C.	260	1319	545	1302	16	728	14	475
P.L.[a]	32	98	448	357				
S.S.[a]	988	322	1046	179				
C.X.[a]	151	<92	149	119				
B.L.[a]	<5	<15	<22	<52				
S.K.[a]	<7	<21	<38	<90				
L.F.[a]	<24	<72	<131	<313				

[a]Simple water facilities.

cysts and oocysts, respectively. The mean parasite concentrations of raw water, treated water and simple facility water samples are shown in Fig. 3. For all three types of water samples, membrane filtration method displayed higher mean concentration of *Giardia* than cartridge filtration method. As for *Cryptosporidium*, the higher mean concentration from the membrane filtration method only occurred in water facility samples. By comparing the parasite concentrations between raw and treated water from five water treatment plants, the mean removal efficiencies of parasites detected by cartridge filtration method were 90.3% for *Giardia* and 76.8% for *Cryptosporidium,* and those by membrane filtration method was 96.5% for *Giardia* and 96.4% for *Cryptosporidium*. The parasite concentration from two filtration methods were correlated as follows: Spearman $R = 0.683$ and $p < 0.001$ for raw water samples; Spearman $R = 0.882$ and $p < 0.001$ for treated water samples; Spearman $R = 0.334$, $p = 0.150$ for simple facilities water samples. In conclusion, considerable variation was found between the occurrence of parasites detected by the two concentration methods, while significant correlation of two methods was discovered between the raw and treated water samples.

Table 2 listed the correlation coefficients between protozoan parasites and indicator microorganisms in the raw water samples. While parasites were concentrated with cartridge filters, the significant correlation was observed between *Giardia* concentrations and the levels of heterotrophic bacteria, total coliforms, fecal coliforms, and Enterococcus. No significant relationship was found between *Cryptosporidium* oocysts and indicator microorganisms besides fecal coliforms. When parasites were concentrated with membrane filters, a significant correlation was observed between *Giardia* concentrations and the levels of heterotrophic bacteria and

Fig. 3. The mean parasite concentrations of two concentration methods from different types of water samples.

Table 2. Nonparametric correlation coefficient between the densities of protozoa to indicator microorganisms (R: Spearman R; P: p-levels; Sample number: 16)

Concentration method	Parasites	Heterotrophic bacteria	Total coliforms	*E. coli*	Fecal coliform	Enterococcus
Cartridge filtration	*Giardia*	$R = 0.794$	$R = 0.657$	$R = 0.229$	$R = 0.700$	$R = 0.505$
		$P < 0.001$	$P = 0.006$	$P = 0.391$	$P = 0.002$	$P = 0.045$
	Cryptosporidium	$R = 0.418$	$R = 0.454$	$R = 0.271$	$R = 0.555$	$R = 0.354$
		$P = 0.107$	$P = 0.077$	$P = 0.309$	$P = 0.025$	$P = 0.178$
Membrane filtration	*Giardia*	$R = 0.715$	$R = 0.394$	$R = 0.154$	$R = 0.642$	$R = 0.456$
		$P = 0.002$	$P = 0.131$	$P = 0.576$	$P = 0.007$	$P = 0.076$
	Cryptosporidium	$R = 0.360$	$R = 0.271$	$R = 0.049$	$R = 0.454$	$R = 0.377$
		$P = 0.170$	$P = 0.309$	$P = 0.856$	$P = 0.077$	$P = 0.149$

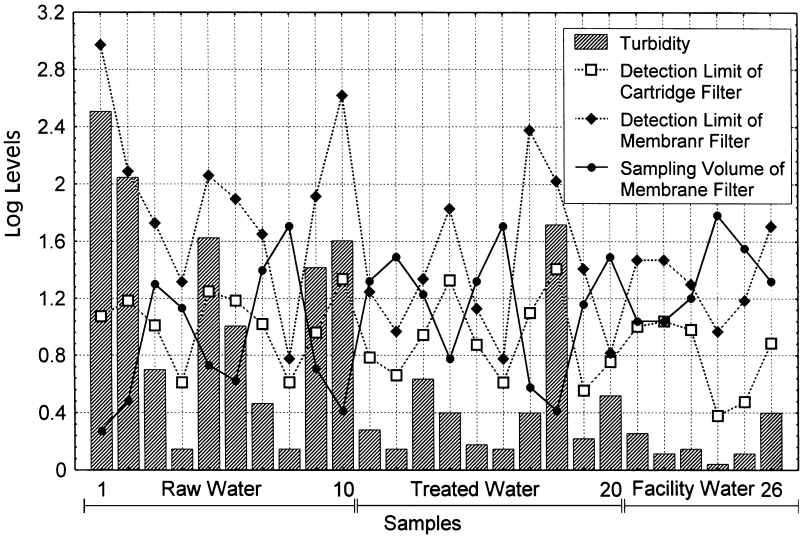

Fig. 4. The distribution of water turbidity, detection limits of two concentration methods and sampling volume of membrane filtration in water samples.

fecal coliforms. No significant relationship was found between *Cryptosporidium* and any indicator microorganism.

The drinking water standard of the USEPA Federal Register (USEPA, 1989) allows one case of microbiologically caused illness per year per 10,000 individuals. To achieve this requirement, Rose et al. (1991) suggested that tap water should not contain more than 7×10^{-4} *Giardia* cysts/100 l on the basis of a geometric mean for one year. Haas and Rose (1995) stated that an outbreak would probably occur if tap water contains more than 10–30 *Cryptosporidium* oocysts/100 l. According to the calculation, the Taiwan water supplies run a high risk for waterborne diseases. However, few outbreaks of giardiasis and cryptosporidiosis were reported. The main reason is that people in Taiwan when choosing tap water as their drinking water, they either boil the water before drinking or treat it with a household purification device. Another reason may be these two parasites are not included in the routine analysis of feces for the diarrhea patients. Therefore, it is hard to tell if any outbreak occurred.

The influence of turbidity on two concentration methods

The mean level of water turbidity was 32 NTU for seeded raw water samples and was 1.2 NTU for seeded treated water samples. Detection of parasite recoveries from seeded water samples (Fig. 1) indicated that any increase in water turbidity resulted in a significant decrease in parasite recovery.

Figure 4 presents the distribution of water turbidity, detection limits of both concentration methods and sampling volume of membrane filtration in all water samples. All of the data in Fig. 4 were derived from log $(X + 1)$ transformation. Results showed similar trends and significant correlation between turbidity and detection limit for membrane filters (Spearman $R = 0.776$, $p < 0.001$) and cartridge filters (Spearman $R = 0.726$, $p < 0.001$). Although membrane filters displayed higher recovery for both parasites in raw and treated water samples than cartridge filters, the sampling volume capacity of membrane filters was significantly reduced by the fact that the membrane pores were easily blocked by the particles of raw water samples.

Therefore, membrane filters were only suitable for water samples under low water turbidity. The significant opposite trend and negative correlation were found between water turbidity level and sampling volume of membrane filters (Spearman $R = -0.706$, $p < 0.001$).

In raw water samples, a significant correlation was discovered between water turbidity and parasites. The correlation coefficients between raw water turbidities and parasite concentrations were 0.435 ($p = 0.092$) for cysts and 0.533 ($p = 0.033$) for oocysts from cartridge filter method, and were 0.589 ($p = 0.016$) for cysts and 0.421 ($p = 0.104$) for oocysts form membrane filters.

CONCLUSIONS

Seeded water samples concentrated by membrane filters achieved higher recovery efficiency than cartridge filters. The detection limits of water samples concentrated by membrane filters were higher than cartridge filters. The cartridge filter can be used for concentrating large volume samples but the membrane filter is for samples of a few liters, especially when the water contains high turbidity. Results of recoveries and detection limit suggested that cartridge filter is suitable for high-turbid water. The membrane filtration method, although safer in preserving parasites during the detection procedure, allows less volume of turbid water to be filtered through the membrane filters and is only suitable for waters of low turbidity and high quality (i.e. filtered water). The occurrences of both parasites and the relationships between parasite concentrations with indicator microorganisms suggest that the detection of parasites by cartridge filtration method is quite different from that of the membrane filtration method.

Though Method 1622 and 1623 have been validated for its high recoveries and low detection limit, it is too expensive to adopt in most countries. Therefore, optimizing the ICR method is very important for developing and under-developing countries. Areas of modification include improving the efficiency in the sample concentration and the accuracy in the detection for parasites in water samples.

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