Cryptosporidium Oocyst Detection in Water Samples: Floatation Technique Enhanced with Immunofluorescence Is as Effective as Immunomagnetic Separation Method

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Abstract: *Cryptosporidium* can cause gastrointestinal diseases worldwide, consequently posing public health problems and economic burden. Effective techniques for detecting contaminated oocysts in water are important to prevent and control the contamination. Immunomagnetic separation (IMS) method has been widely employed recently due to its efficiency, but, it is costly. Sucrose floatation technique is generally used for separating organisms by using their different specific gravity. It is effective and cheap but time consuming as well as requiring highly skilled personnel. Water turbidity and parasite load in water sample are additional factors affecting to the recovery rate of those 2 methods. We compared the efficiency of IMS and sucrose floatation methods to recover the spiked *Cryptosporidium* oocysts in various turbidity water samples. *Cryptosporidium* oocysts concentration at 1, 10^1, 10^2, and 10^3 per 10 μl were spiked into 3 sets of 10 ml-water turbidity (5, 50, and 500 NTU). The recovery rate of the 2 methods was not different. Oocyst load at the concentration < 10^2 per 10 ml yielded unreliable results. Water turbidity at 500 NTU decreased the recovery rate of both techniques. The combination of sucrose floatation and immunofluorescense assay techniques (SF-FA) showed higher recovery rate than IMS and immunofluorescense assay (IMS-FA). We used this SF-FA to detect *Cryptosporidium* and *Giardia* from the river water samples and found 9 and 19 out of 30 (30% and 63.3%) positive, respectively. Our results favored sucrose floatation technique enhanced with immunofluorescense assay for detecting contaminated protozoa in water samples in general laboratories and in the real practical setting.

Key words: *Cryptosporidium,* oocyst, floatation technique, immunomagnetic separation, immunofluorescence asaay, water

INTRODUCTION

Cryptosporidium is a protozoan parasite capable of infecting many mammalian species, including humans. It causes watery diarrhea worldwide. In the 1993 Milwaukee cryptosporidiosis outbreak, 403,000 people were infected and 110 died [1]. Healthy individuals are relatively unaffected but the infection is life threatening in immunocompromised hosts, such as HIV patients. The mortality rate of cryptosporidiosis in immunocompromised individuals reaches 50% [2], and so far, no definitively effective drug is available. Infection has been known to occur from as low as 10-100 oocysts, whilst only 1 g of feces from infected animals in the laboratory contained 10^6 oocysts [2,3]. The efficiency and suitable techniques of detection are important in preventing and controlling water contamination by this organism.

There are many techniques for detecting contaminated *Cryptosporidium* oocysts. High efficiency and reasonable cost are important factors in the selection of the technique. Recently, immunomagnetic separation (IMS) method recommended by US Environmental Protection Agency (US-EPA) is widely used due to it high efficiency, rapid process, and requirement of less examiner's experiences than other techniques. The oocyst is separated from other organisms and debris by specific antibody coated-magnetic bead and is then identified with immunofluorescense assay technique (FA) under fluorescence microscope. However, this method still has some limitations, such as cross reaction of antibodies and water turbidities [1,4]. Moreover, the major disadvantage of this method is its expensive cost which is not affordable by many laboratories. Sucrose floatation technique is generally used to separate different organisms by appropriate specific gravity. It is easy to perform, effective, and relatively cheap, but it required experienced examiners to differentiate the oocysts from other organisms that might be floated as well. Flotation method is time consuming. In addition, not only

the mentioned limitations but also other factors, such as organism load and water turbidity, affect the recovery rate of IMS and flotation methods. Although these 2 methods have been studied [5-13], no comparison of their efficiency for recovering the spiked *Cryptosporidium* oocysts in various turbidity water samples was reported. We, therefore, undertook the present study.

MATERIALS AND METHODS

Various water turbidities

Suspended particles in water from Chao Phraya River, Thailand, were collected, washed by distilled water and centrifuged 3 times at 3,500 rpm for 10 min. Cleaned particles were added to distilled water to make 3 sets of water turbidities at 5, 50, and 500 NTU (Nephelometric Turbidity Unit) measured by 2,100P portable turbidimeter (Hach Company, Loveland, Colorado, USA). Each set of turbidities contained 4 tubes with 10 ml volume.

Spiked *Cryptosporidium* oocyst preparation

Cryptosporidium oocysts were produced at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Thailand. This project was approved by the Faculty of Tropical Medicine, Animal Care and Use Committee (FTM-ACUC007/ 2006). Briefly, 3-4 day old mice were orally infected with 100,000-120,000 *Cryptosporidium* oocysts. On day 8 after infection, mice were euthanized and the intestinal parts were collected. A simple extraction procedure was used and purified using Ficoll gradient centrifugation. After purification, oocysts were preserved in PBS/antibiotics at 4°C before use. The oocysts were enumerated by hematocytometer to prepare 4 sets of oocyst concentrations, 1, 10^1, 10^2, and 10^3, and then spiked into each set of water turbidities as mentioned above.

Immunomagnetic separation (IMS)

We used Dynalbeads GC-Combo kit (Dynal Biotech, Oslo, Norway) and followed manufacturer's instructions. Briefly, a 10 ml of each sample was transferred separately into L10 tube. Then, 1 ml of 10X SL-buffer A and 10X SL-buffer B (from the kit) were added, followed by addition of 100 μl anti-*Cryptosporidium* beads, and then the tube was rotated at 18 rpm for 1 hr. One ml of 1X SL-buffer, prepared by PBS: 10X SL-buffer A = 9 : 1, was added into the sample. Each tube of sample was held with MCP-1 magnet and mixed slowly by hand until the pellets at the bottom of the tube were attached to MCP-1 magnet then cleared solution of samples was removed carefully by Pas-

teur pipette without touching the attached pellets. Only attached pellets were remained in the L10 tube. The L10 tube was removed from MCP-1 magnet and then 1 ml of 1X SL-buffer A was added to detach the pellets from the tube. Those pellets were pipetted into an eppendorf tube and then held with MPC-S holder. The magnet was inserted into an MPC-S holder, and then mixed well 10 times. Cleared solution was removed and only the pellets were left in the eppendorf tube. A 100 μl of 0.1 N HCl was added into each eppendorf tube for separation of oocysts from magnetic beads and vortexed thoroughly. This eppendorf tube was incubated at room temperature for 5 min, and then held with inserted magnet MPC-S holder again. Cleared solution containing oocysts, if any, was removed into a new eppendorf tube. A 10 μl of 0.1 N of NaOH was added for neutralization. The solution was kept at 4°C until immunofluorescence assay (FA) was performed.

Sucrose floatation

Each water sample was mixed with sucrose solution with specific gravity of 1.18 in a 10 ml beaker, then poured into a 20 ml-volume cylinder container with wide surface area of 5 cm diameter and left for 10 min. The sample was covered with a plastic sheet of equal size to the surface area of the container and left for 20 min. The plastic sheet was carefully removed and rinsed into a new beaker. The same plastic sheet was reused and the same process was repeated thrice. The total solutions after rinsing plastic sheet were combined and centrifuged at 3,500 rpm for 10 min, and then the supernatant was removed. Two ml of distilled water was added and thoroughly mixed with the pellet. A 25 μl of each sample was examined under light microscope at × 400 magnification. For enhancing oocyst identification, immunofluorescence assay (FA) technique was performed on floated samples as well.

Immunofluorescence assay (FA)

Giardia-Cryptosporidium CEL IF Test (Celllabs, Australia) kit was used. According to the kit instructions, each sample from IMS and sucrose floatation processes was smeared on well slides, and dried overnight. Each slide was fixed with acetone for 5 min. The sample was covered by 25 μl of Crypto Cel Reagent (RR2) (performed without light exposed), and then incubated at 37°C in a humid chamber for 30 min. The slide was rinsed gently with a bath PBS for 1 min. The microscope slide was mounted by 25 μl of RMG solution, and covered with a cover slip. The entire specimen was examined under fluorescence microscope

at $\times$ 400 magnification.

Application in practical situation

We collected 20 L water samples from Chao Phraya River 1 hr before the drainage pump at the Department of Drainage and Sewerage of Bangkok Metropolitan was operated and another soon after the drainage pump was turned on. Three more samples, each 20 L, were also collected 2, 4, and 6 hr later. The other further 5 sets of water samples were similarly taken at other times depending on drainage pumping operation. Altogether we collected 30 samples and recorded their turbidity, temperature, and pH. The collected samples were filtered through 0.1 diameter pore filter paper. The sediments were then washed and kept in distilled water for further sucrose floatation and immunoflorescence assay, as aforementioned, to identify *Cryptosporidium* and *Giardia* contamination.

RESULTS

Table 1 shows the recovery efficiency in spiked water samples, by immunomagnetic separation technique plus fluorescence assay (IMS + FA) and sucrose floatation enhanced with fluorescent assay (SF + FA) in difference water turbidities. At the oocyst concentrations of 1 and 10^1, IMS + FA did not recover any *Cryptosporidium* from the spiked sample in all turbidities of 5, 50, or 500 NTU waters. However, IMS + FA detected 2 and 5 oocysts per 25 μl at the concentrations of 10^2 and 10^3 in the turbidity of 5 NTU water, respectively. In more turbid waters, the numbers of recovered oocysts by IMS + FA were reduced to 1 and 3 oocysts at the concentrations of 10^2 and 10^3 in 50 NTU water, respectively, whereas in 500 NTU turbidity water, 4 oocysts were

found only at 10^3 concentration of spiked sample. Therefore, the recovery efficiency of IMS + FA was 50%, 50%, and 25% at the turbidity of 5, 50, and 500 NTU, respectively. SF + FA recovered oocysts at a lower concentration (10^1) than IMS + FA, even though only 1 oocyst was found in 5 NTU turbidity water, and the recovery efficiency was 75% when combined with the other 2 positive samples at the concentrations of 10^2 and 10^3. In higher turbidity waters of 50 and 500 NTU, SF + FA showed 50% recovery efficiency and could also detect more oocysts than IMS + FA, i.e., 5 and 8 oocysts versus 1 and 3 oocysts at 10^2 and 10^3 concentrations; 2 and 6 oocysts versus 0 and 4 oocysts at 10^2 and 10^3 concentrations, respectively (Table 1). The spiked oocysts lower than the concentration of 10^2 per 10 ml showed a low recovery yield. Water turbidity influenced the recovery efficiency in both methods.

In the field situation, we found 9 out of 30 (30%) river water samples positive for *Cryptosporidium*, and 63.3% (19 out of 30) were positive for *Giardia*. The mean temperature difference of all 6 groups of water samples were minimal varying from 26.9 to 27.3℃, while the mean pH was 6.64-6.91. Water turbidity increased according to the sewage drainage to the river, revealing 2 highest peaks soon after and at 2 hr after pumping which correlated with the highest number of both protozoa (Table 2). *Giardia* cysts were found more than *Cryptosporidium* oocysts in 20 L of river water.

DISCUSSION

The recovery yield of *Cryptosporidium* oocysts from contaminated water depends on the purification and identification methods used. Purification by IMS, including identification by immunofluorescence assay (FA) recommended by the US-EPA method 1622 in 2001 has been widely used with recovery rates varying from 40.0 to 100% [5-8,10]. Apart from IMS, density-based purification methods, such as sucrose floatation and Percoll-sucrose centrifugation were also applied for purifying oocysts from very turbid water samples [9,11,14]. In the present spiked water sample study, we found that sucrose floatation method enhanced with immunofluoresence assay (SF-FA) showed slightly higher purification efficiency than IMS-FA, especially in high turbidity waters. These findings were supported by Feng [1] who showed that when water turbidity increased from 5 to 40%, the recovery rate of IMS method decreased from 58.0% to 24.9%. The pH was another factor affecting the recovery yield by IMS. Kuhn et al. [15] studied the effects of pH on suspended oocysts

Table 1. Comparison of recovered oocysts by immunomagnetic separation and sucrose floatation technique enhanced with immunofluorescence assay

No. of spiked oocysts per 10 ml water	No. of recovered oocysts per 25 μl in various water turbidity					
	5 NTU		50 NTU		500 NTU	
	IMS-FA	SF-FA	IMS-FA	SF-FA	IMS-FA	SF-FA
1	-	-	-	-	-	-
10^1	-	1	-	-	-	-
10^2	2	2	1	3	-	2
10^3	5	6	3	8	4	6
Recovery efficiency	50%	75%	50%	50%	25%	50%

NTU, nephelometric turbidity unit, a measurement unit for water turbidity; IMS, immunomagnetic separation technique; FA, immunofluorescence assay; SF, sucrose floatation technique.

Table 2. Detection of *Cryptosporidium* oocysts and *Giardia* cysts by sucrose floatation and fluorescence assay (SF-FA) from 30 water samples collected from Bangkok Cha Phraya River before and after drainage pumping

Group of samples*	Turbidity (NTU) Mean (min-max)	Temperature (°C) Mean (min-max)	pH Mean (min-max)	Results			
				Cryptosporidium oocysts		*Giardia* cysts	
				No. positive samples/total	Mean (min-max)/20 L	No. positive samples/total	Mean (min-max)/20 L
1	30.6 (20.9-47.2)	27.1 (26-29)	6.86 (6.8-7.1)	2/6	1.7 (0-5.6)	4/6	4.9 (0-13.6)
2	46.5 (36.0-53.8)	27 (26-28)	6.83 (6.7-7.0)	2/6	5.8 (0-6.0)	5/6	16.6 (0-41.6)
3	69.7 (44.8-112)	26.9 (26-28)	6.77 (6.7-6.9)	3/6	7.7 (0-18.4)	6/6	23.9 (5.2-50.8)
4	51.2 (37.8-70.1)	27.2 (26-28)	6.64 (6.7-7.9)	1/6	1.5 (0-8.8)	2/6	2.4 (0-14.4)
5	47.1 (21.2-60.6)	27.3 (26-28)	6.91 (6.7-7.2)	1/6	2.1 (0-12.8)	2/6	9.1 (0-38.4)
Protozoa contamination in surface water samples				9/30 (30%)		19/30 (63.3%)	

*Group 1 = samples collected 1 hr before the drainage pumping was operated.
Group 2 = samples collected soon after the drainage pump was turned on.
Group 3, 4, 5 = samples collected at 2, 4 and 6 hr after the drainage pump was operated, respectively.

recovery in deionized (DI) water. They found that the water sample at pH 7.0 yielded the highest recovery rate (96.2%) by IMS method. When they increased the water pH up to 7.5, the recovery decreased to 51.2%. They also decreased the pH of water to 6.5, the recovery was also decreased to 49.5%. Their results indicated that the optimal pH of water samples for using this method was 7.0. Our spiked water samples tended to be acidic at pH 6.6 (data not shown), therefore, this might be the reason why the recovery rate by IMS was lower than in other studies. Moreover, dissolved iron and iron-like particles which were commonly found in both surface and underground waters were considered as interferences of IMS method. Yakub and Knauer [16] found that dissolved iron concentration of > 4 mg/L in water sample could decrease the recovery by this method. When dissolved iron and iron-like particles were removed or treated, the pH of water was increased. In addition, the water pH affected antibody affinity in IMS method, so antigen-antibody cross reaction might decrease the recovery rate. As mentioned above, in certain situations using IMS purification method need special precaution. Moreover, its expensive cost also limits the affordability of this method. However, it had some advantages, such as rapidity in processing and less personnel skill required than sucrose floatation technique.

We compared and found that sucrose floatation technique was much cheaper and easier to perform and resulted in good purification yield. Using sucrose floatation when IMS method is not suitable is an alternative way, but identification by fresh smear under light microscope is time consuming, requiring highly skilled personnel and low sensitivity detection. To overcome those obstacles, Fujino et al. [3] suggested that sucrose solution with specific gravity of 1.266 yielded higher recovery rate than

that with a lower specific gravity and floated oocysts were deep pink in color. We included the immunofluorescence assay to enhance oocyst visualization and confirmation. Nevertheless, oocyst-like objects or some algae either autofluoresce or cross react with commercial antibodies yielding false positives [17]. Oocyst identification by FA staining is thus recommended for confirmation by other methods. Furthermore, we have applied our study by using a wide surface area and short height container for floating process. By doing so, the floating time of the oocysts was shortened resulting in higher recovery yields. However, this method might work only in the laboratory but might not work in practice. Our further study using SF-FA in the river water with sewage drainage showed good results and found that the prevalence of *Cryptosporidium* oocysts and *Giardia* cysts contamination were similar to a previous study [18] indicating that it's practicable.

In conclusion, we suggest using sucrose floatation technique enhanced with FA for detecting *Cryptosporidium* and *Giardia* oocysts in water samples especially in high water turbidity, low or high water pH, and high iron particle interference in both laboratory-based and practical situations. It was more economical and highly efficient as the IMS technique. We also suggest applying a wide surface and short-height container for floating.

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