Performances of the Immunomagnetic Separation Method for *Cryptosporidium* in Water under Various Operation Conditions

Bing-Mu Hsu[†,‡] and Chihpin Huang[*,§]

Department of Environmental Engineering and Health, Yuanpei Institute of Science and Technology, 306 Yuanpei Street, Hsinchu, Taiwan, Republic of China, Department of Medical Research, Chiayi Christian Hospital, 539 Chung-Shiao Street, Chiayi, Taiwan, Republic of China, and Institute of Environmental Engineering, National Chiao Tung University, 75 Po-Ai Street, Hsinchu, Taiwan, Republic of China

Immunomagnetic separation (IMS) has been specified as a standard method for the measurement of *Cryptosporidium* in some countries. In this study, the IMS method was evaluated on the basis of the recovery efficiencies of *Cryptosporidium* oocysts at various IMS operation conditions. The average recovery for different *Cryptosporidium* concentrations in deionized water was 82.6 ± 18.2% (n = 52). No significant change in recovery was observed by altering the debris ratio of the water samples. The efficiency was increased by prolonging the reaction time, and by increasing the amount of immunomagnetic beads. The recoveries of oocysts seeded in an Eppendorf with a small reaction volume were similar to those seeded in glass tubes with 10 times the reaction volume. The recovery efficiency of oocysts was reduced significantly when the reaction buffer was replaced by PBS. In conclusion, this method has good reproducibility and high recovery.

Introduction

Cryptosporidium has been recognized as a common pathogenic protozoan of the gastrointestinal tract of many vertebrates. Many outbreaks of human cryptosporidiosis have been reported in the last few decades. From 1984 to 1994, 19 outbreaks of cryptosporidiosis were reported in the United States. During 1986−1995, 21 outbreaks were reported in the United Kingdom (*1*). The great attention on *Cryptosporidium* is due to not only the recent outbreaks but also its resistance to inactivation by chlorine (*2*). This protozoan may become the focal organism for drinking water regulations (*3*). Despite the difficulties in analytical procedures, it has generally been agreed that the average amounts of *Cryptosporidium* are from 0.005 to 252.7 oocysts/L for surface waters (*4*, *5*).

The occurrence of *Cryptosporidium* in drinking water has brought about an increased need for detection at levels concerning human health. The method available for the detection of *Cryptosporidium* oocysts in water samples involves three stages: sample collection and concentration, separation of oocysts from other debris, and detection of the oocysts. The fluorescent antibody procedure specified in the Information Collection Rule (ICR) of the United States is the first standard method for detecting *Cryptosporidium* in water samples (*6*). This ICR method, however, has been heavily scrutinized (*7*, *8*). A major drawback is that only a small fraction of the sample volume collected can be analyzed, which results in low recovery and low precision. Methods 1622 and 1623, due to improved filtration procedures and adoption of immunomagnetic separation (IMS), are expected to have a higher recovery and lower detection limit. The estimated method detection limit of 10 oocysts/L typifies the minimum concentration of oocysts that can be detected in a 10 L sample with no interferences present (*9*, *10*). IMS consists of two stages. In the capture stage, paramagnetic beads coated with antibodies against *Cryptosporidium* oocysts react to epitopes on the outer wall of the oocysts. In the separation stage, the conjugated magnetic oocysts are separated from debris by a magnet (*11*).

The aims of this study were to evaluate the IMS method and to compare the recovery efficiency under this method in different samples. The recovery efficiencies of various operation conditions, including the reaction time, reaction volume, sample turbidity, use of buffer, and dosage of oocysts and immunomagnetic beads, were evaluated. The recommendations for improving the recovery of the IMS method were also included in this study.

Materials and Methods

Preparation of an Oocyst-Containing Sample. In this study, *Cryptosporidium parvum* oocysts, obtained from Pleasant Hill Farms (Idaho), were used. The *C. parvum* oocysts can react with the antibodies of Hydrofluor-Combo *Giardia/Cryptosporidium* and the Dynabeads GC-Combo Kit. The oocyst concentration of the purchased stock preparation was about 10^6 oocysts/mL. The stock preparation was mixed and diluted to concentrations between 10^3 and 10^5 oocysts/mL with 0.1% PBS. The concentration of oocysts in the diluted stock solution was examined using the immunofluorescence assay technique. To do this, the diluted stock solution was mixed thoroughly and diluted to about 5×10^3 oocysts/mL. A 40−60 μL portion of the diluted solution was then pipetted onto the glass slides (Dynal Spot-On), dried at

* To whom correspondence should be addressed. Fax: 886 3 5725958. E-mail: cphuang@cc.nctu.edu.tw.
† Yuanpei Institute of Science and Technology.
‡ Chiayi Christian Hospital.
§ National Chiao Tung University.

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room temperature, stained with fluorescent-labeled antibodies (Hydrofluor-Combo *Giardia*/*Cryptosporidium*; Ensys, Inc., North Carolina), and counted via fluorescence microscopy. The staining protocol was described as follows: A 50 μL portion of methanol was added to each well of the slide and allowed to completely dry at room temperature. A 60 μL portion of the primary antibody reagent at working dilution was applied to each well of the slide, which was covered completely. The slide was placed in an incubator at 37 °C for 25 min. After incubation, the primary antibody reagent was gently aspirated from the well by absorbent paper. A 60 μL portion of the deionized water was added to each well and left for 1−3 s, and the water was then aspirated from each well. This washing cycle was repeated five times to remove the residual antibodies. A 60 μL portion of the labeling reagent at working dilution was applied to each well of the slide. The subsequent incubation and washing steps are the same as those used with the primary antibody reagent. After the final washing, 10 μL of DABCO/glycerol mounting medium was added to each well, and a cover slip was applied to each slide.

The deionized water and the concentrated raw water samples were seeded with various numbers of oocysts by adding a specific volume from the diluted stock solution. The raw water samples were taken from Cheng-Ching Lake located in southern Taiwan. Particle pellets were collected from the water by filtration through Envirochek (Pall Gelman Sciences, Michigan) and then were eluted from the Envirochek by a shaker. The eluate was concentrated by centrifugation. The debris was washed and mixed with deionized water to prepare samples of various turbidities. The original quantity of the oocysts in the debris was less than 1 oocysts/0.5 mL of debris.

IMS Procedures. The IMS was operated following the instructions provided by the Dynal Co. (Dynabeads GC−Combo Kit; Dynal A.S, Oslo, Norway). Aliquots of Dynabeads and 10× SL Buffer A/B were added to the water sample and then incubated on a rotary shaker at 15 rpm. The bead−oocyst complexes were first captured by a magnetic particle concentrator (MPC-1 or MPC-M). The supernatant was discarded, and the magnet was removed. The bead−oocyst complexes were resuspended in the solution of 1× SL Buffer A, transferred to an Eppendorf, and then recaptured by a smaller sized magnetic particle concentrator (MPC-M). Aliquots of HCl (0.01 N) were added to dissociate the protozoa from the beads. The protozoa-containing solution was neutralized with NaOH (0.1 N), transferred to a glass slide (Dynal Spot-On), stained with fluorescently labeled antibodies (Hydrofluor-Combo *Giardia*/*Cryptosporidium*; Ensys), and enumerated by the epifluorescent microscope (Olympus, Japan).

Testing Procedures. To investigate the applicability of IMS under various operation conditions, we designed the evaluation study by manipulating several parameters related to the existing IMS procedures. These parameters include the reaction time, reaction volume, sample turbidity, use of buffers, and dosage of oocysts and immunomagnetic beads. The schematic of the IMS procedures and operating conditions tested in our study are shown in Figure 1. First, we compared the recovery efficiencies between the Eppendorf containing 1.2 mL of the sample and the glass tubes (Dynal L10 tubes) containing 12 mL of the sample. To determine the recovery efficiency for different numbers of oocysts, we varied the seeding concentration from 32 to 2.0 × 10^5 oocysts/mL of deionized water. To study the effect of the turbidity of the water samples, deionized water and turbid samples containing 1/40, 1/20, 1/10, and 1/5 debris were also detected. To examine the influence of the reaction time on the recovery efficiency, four different reaction times (0.5, 1.0, 1.5, and 2.0 h) were tested. Different dosages of magnetic beads (water samples containing 5/10000, 1/1000, 2/1000, 4/1000, 6/1000, 8/1000, and 1/100 immunomagnetic beads, respectively) and different buffers (10× PBS and 10× SL Buffer A/B) were also explored for their influence on the IMS performance. The number of oocysts observed on each slide was taken as the number of oocysts per milliliter of sample. The recovery efficiency was calculated from the number of oocysts seeded in the sample and that remained after the IMS procedure.

Results and Discussion

Effects of the Oocyst Concentration. In this study, various concentrations of oocysts ranging from 32 to 2.0 × 10^5 per milliliter were seeded into the deionized water, and their recovery efficiencies are shown in Figure 2. The average recovery for these samples was 82.6 ± 18.2% (n = 52), ranging from 70.6% to 91.2%. The results indicated that the dosage of 1/100 immunomagnetic beads recommended by method 1623 is sufficient to conjugate a large number of oocysts. The highest recovery efficiency was found at the seeding concentration of 800 oocysts/mL, and the lowest level was found at 64 oocysts/mL. Figure 2 also shows that there are no significant differences between the mean recovery efficiencies for the entire range of oocyst concentration. The inaccuracy in oocyst counting and the deficiency in the experimental procedure may account for the large deviation.

Effects of the Sample Turbidity. The IMS kit advocates the use of water samples containing a debris ratio of less than 1/20. To understand the influence of turbidity on recovery efficiencies, deionized water (0 NTU) and turbid samples containing 1/40 debris (2500 NTU), 1/20 debris (5000 NTU), 1/10 debris (10000 NTU), and 1/5 debris (20000 NTU) were seeded with 800 oocysts/mL. As shown in Figure 3, there is no significant difference in recovery among various turbid samples. The average recovery efficiencies for each turbid sample ranged from 74.8% to 80.1%. The average recovery of all turbid samples was 79.4% (n = 20), which was lower than that of deionized water. Di Giovanni et al. (*12*) obtained a similar result. Hence, it is evident that the water turbidity lowers the parasite recovery, which may be due to the interference by the particles and other microorganisms. However, the ratio of debris does not have much impact on the degree of interference. A possible explanation is that there is a high specific conjugated bond between the antibodies coated on the magnetic beads and the epitopes on the outer wall of *Cryptosporidium*.

Effects of the Amount of Immunomagnetic Beads. The effect of the dosages of immunomagnetic beads on oocyst recoveries was evaluated on the deionized water containing 800 oocysts/mL. The results in Figure 4 show that there was a significant correlation between the recovery efficiency and the number of immunomagnetic beads in the samples (r = 0.985, p < 0.02, n = 7). The explanation may be that the recovery is increased by more frequent collisions, which are enhanced by greater numbers of immunomagnetic beads.

Effects of the Reaction Time. The influence of the reaction time on the recovery efficiencies in deionized

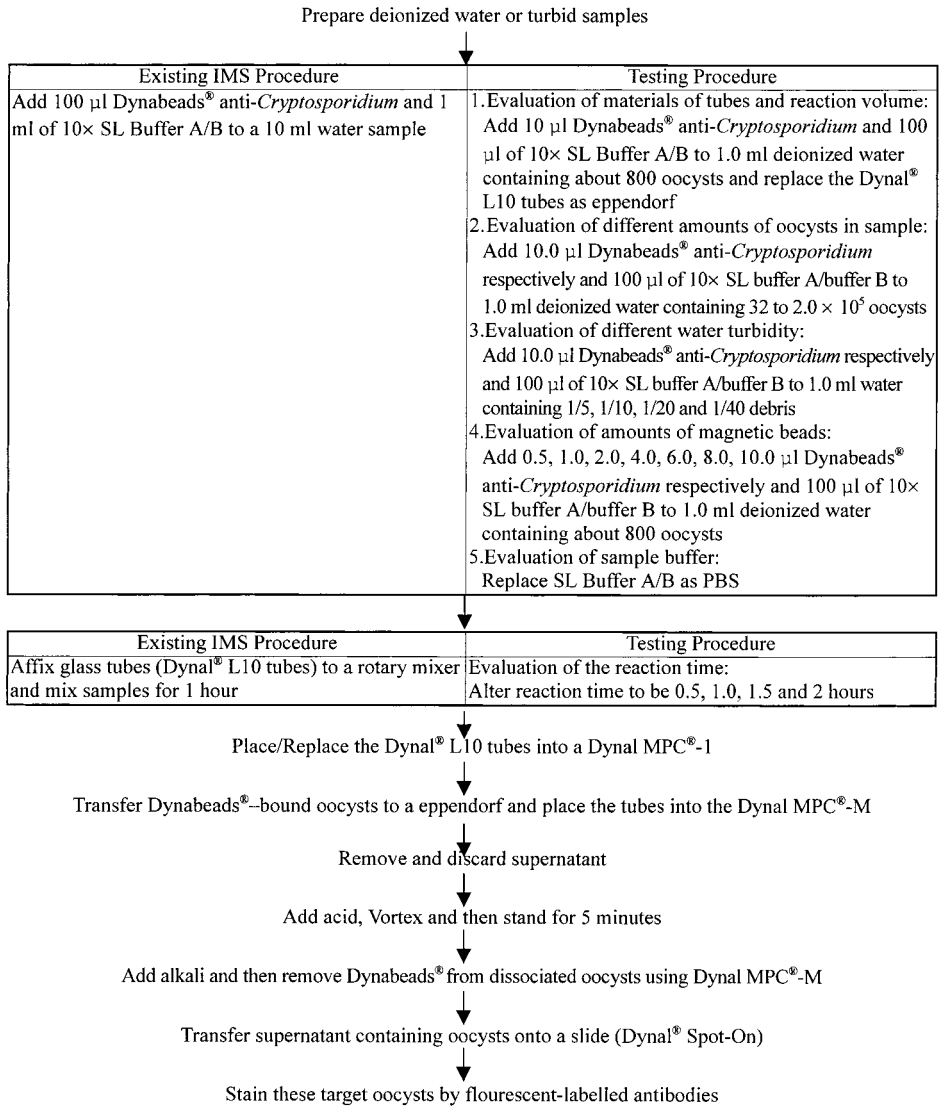

Figure 1. IMS and testing procedures for separation of *Cryptosporidium* from water samples.

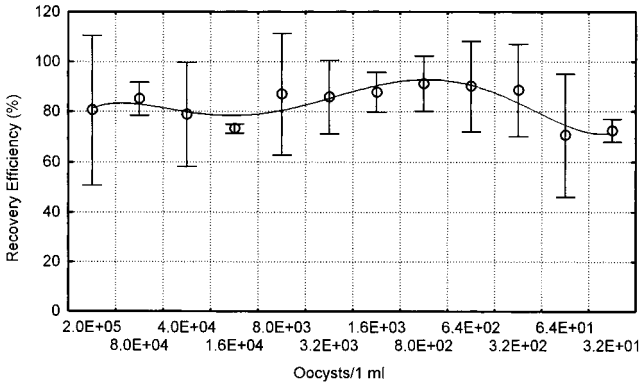

Figure 2. Average recovery efficiencies and their 95% confidence levels for deionized water containing various concentrations of oocysts.

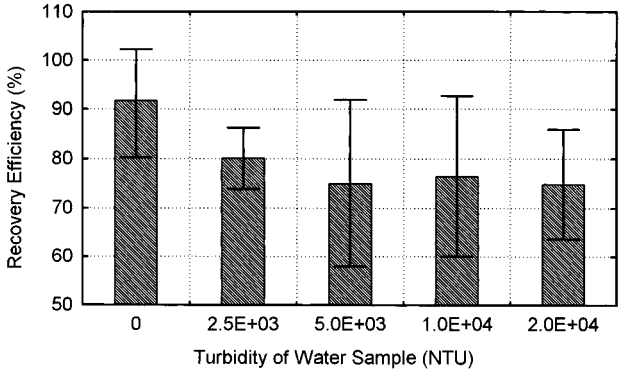

Figure 3. Average recovery efficiencies and their 95% confidence levels for deionized water and turbid samples containing 800 oocysts/mL.

water samples is presented in Figure 5. It is obvious that the recovery efficiencies were increased by the reaction time. However, the increasing rates in the three time intervals were different. A 1 h reaction time exhibits double recovery compared to a $^1/_2$ h reaction time. When the reaction time is over 1 h, the increasing rate drops significantly. The increasing rate was about 4.0% when the reaction time was between 1.0 and 1.5 h. When the reaction time was prolonged from 1.5 to 2.0 h, the increasing rate accelerated to 4.9%. It is obvious that the reaction time is a key factor in improving the recovery efficiency of IMS, and the critical reaction time is about 1 h.

Effects of the Sample Buffer. To investigate the effect of buffer on conjugation, two buffer reagents (e.g., PBS and SL Buffer A/B) were added to the test samples separately. We chose PBS as it is commonly used in immunoreactions. SL Buffer A/B, recommended by

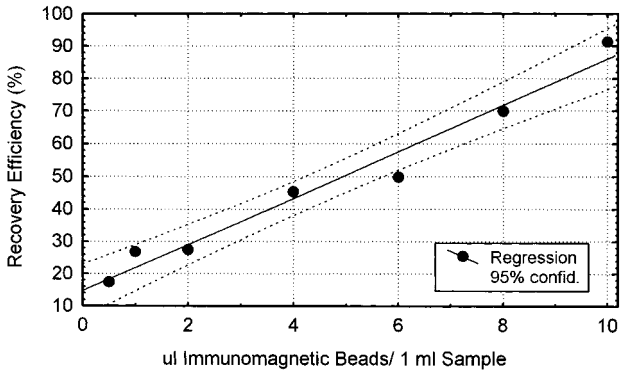

Figure 4. Relationships between the dosages of immunomagnetic beads and the corresponding recovery efficiencies in deionized water containing 800 oocysts/mL (regression line $y = 7.1310x + 14.696$; $r = 0.985$, $p < 0.02$).

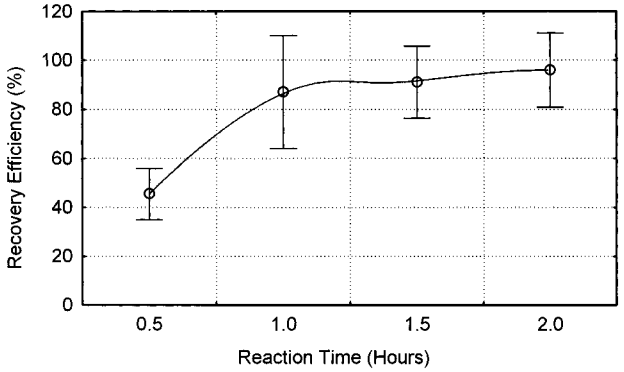

Figure 5. Trends of recovery efficiencies for deionized water and their 95% confidence levels at four reaction times.

the Dynal Co., was used for comparison. The results in Figure 6 show that the average recovery of oocysts in SL Buffer A/B was 91.7 ± 18.7% ($n = 9$) and that in PBS was 68.2 ± 10.4% ($n = 4$) when 800 oocysts/mL were seeded. The samples in SL Buffer A/B achieved a higher recovery efficiency by a margin of 23.5%.

Evaluation of Different Lot Numbers of the Dynabeads Kit. To evaluate the consistency of the Dynabeads Kit for the oocyst recovery efficiency, five cases of the Dynabeads GC−Combo Kit from different lot numbers were tested while 800 *Cryptosporidium* oocysts/mL were seeded in the deionized water. The testing results showed that the average recovery was 83.4% with a standard deviation of 12.9% ($n = 5$).

Effects of the Vessel Volume and Material. Two reaction vessels, Eppendorf and Dynal L10 tubes, were compared with respect to their relationship with their recoveries of oocysts. The reaction volume in the Dynal L10 tube was 12 mL, and that in the Eppendorf was 1.2 mL. The same concentration of oocysts was used in both cases. The results in Figure 7 show that, with deionized water, the average recovery was 82.6 ± 18.2% ($n = 52$) for the Eppendorf and 74.7 ± 7.1% ($n = 3$) for the Dynal L10 tube. For samples containing debris, the average recovery was 76.6% ($n = 20$) for the Eppendorf and 68.0% ($n = 13$) for Dynal L10 tube. Bukhari et al. (*13*) have reported similar recoveries ranging from 68% to 83.2% for deionized water and lower recoveries from 35% to 70% for turbid samples (5000 NTU) when the same IMS kit was used. A much wider variation was discovered by Campbell and Smith (*11*) for the recovery in deionized water, which ranged from 58.2% to 147.4% and from 4.8% to 109.1% while the sample turbidity was about 610 NTU. Since the increase in reaction volume did not

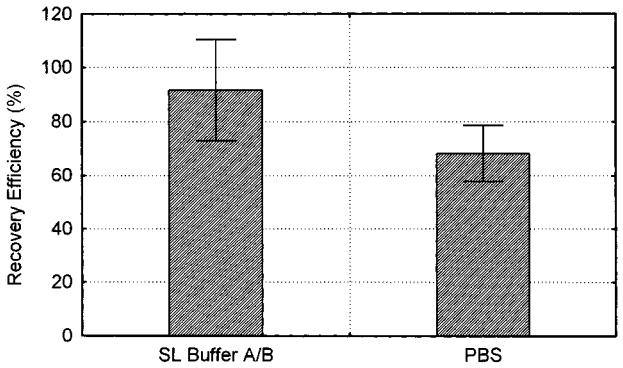

Figure 6. Recovery efficiencies and their standard deviations for deionized water containing 800 oocysts/mL prepared in 1× SL Buffer A/B or 1× PBS.

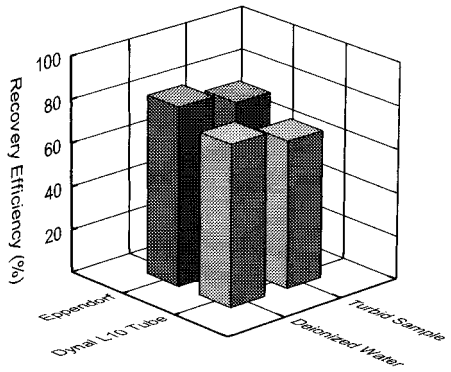

Figure 7. Recovery efficiencies of deionized water and turbid samples in two reaction vessels.

improve the recovery efficiencies, a smaller reaction volume and vessels may be adopted to reduce the cost of the kit for evaluating the performance of the kit on the unknown samples.

Conclusion

The IMS procedure has higher and more stable recovery efficiencies than previously recognized. The average recovery efficiency was 82.6 ± 18.2% ($n = 52$) for deionized water. The average recovery efficiencies of four turbid samples ranged from 74.8% to 80.1%. The recovery efficiencies were affected by the use of buffer, dosage of immunomagnetic beads, and reaction time. The debris ratio and oocyst concentration have a very limited effect on the recovery. The recovery increased when the dosage of Dynabeads was raised. Reducing the reaction volume to raise the concentration of Dynabeads and the debris ratio in the samples would benefit the IMS performance. The recovery efficiencies could be improved by prolonging the reaction time. The evaluation indicated that over 1 h of reaction time was sufficient. The lower recovery for using PBS indicated that PBS should not replace SL Buffer A/B. The glass tube with a larger reaction volume displayed a lower recovery than the Eppendorf. A small reaction volume with a 1/100 volume ratio of immunomagnetic beads is recommended for evaluation of IMS recoveries for oocysts from an unknown sample.

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