Evaluation of Cyst Loss in Standard Procedural Steps for Detecting of *Giardia lamblia* and *Cryptosporidium parvum* in Water

Kyung-Ju Kim, Hyang-Hee Jung, and Kisay Lee*

School of Biotechnology and Environmental Engineering, Myongji University, Yongin 449-728, Korea

Abstract The standard procedure outlined by the United States Environmental Protection Agency (US EPA) in Method 1623 for analyzing *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in water samples consists of filtration, elution, centrifugal concentration, immunomagnetic separation (IMS), and immunofluorescence assay (IFA) followed by microscopic examination. In this study, the extent of (oo)cyst loss in each step of this procedure was evaluated by comparing recovery yields in segmented analyses: (i) IMS + IFA, (ii) concentration + IMS + IFA, and (iii) filtration/elution + concentration + IMS + IFA. The complete (oo)cyst recovery by the full procedure was 52~57%. The (oo)cyst loss in the IMS step was only 0~6%, implying that IMS is a fairly reliable method for (oo)cyst purification. Centrifugal concentration of the eluted sample and pellet collection before IMS resulted in a loss of 8~14% of the (oo)cysts. The largest (oo)cyst loss occurred in the elution step, with 68~71% of the total loss. The permeated loss of (oo)cysts was negligible during filtration of the water sample with a 1.0-μm pore polyethersulfone (PES) capsule. These results demonstrated that the largest fraction of (oo)cyst loss in this procedure occurred due to poor elution from the filter matrix. Improvements in the elution methodology are therefore required to enhance the overall recovery yield and the reliability of the detection of these parasitic protozoa.

Keywords: *Cryptosporidium parvum*, *Giardia lamblia*, detection and analysis, Method 1623, (oo)cysts, recovery loss

Giardia lamblia and *Cryptosporidium parvum* are the major parasitic protozoa that are transmitted to humans as cysts or oocysts through both untreated and treated water. *Giardia* cysts are oval-shaped and 10~16 μm in diameter; *Cryptosporidium* oocysts are spherical and 4~8 μm in diameter. Since their presence in water has led to frequent outbreaks of giardiasis and cryptosporidiosis in many countries, methodologies for monitoring and removing these protozoan (oo)cysts from the water supply are of great concern for public health [1,2].

Several processes have been developed to improve the accuracy and reliability of detecting these protozoan (oo)cysts. One of the most popular methods is Method 1623, developed by the US EPA [3,4]. The procedure, as outlined in Method 1623, to detect *G. lamblia* cysts and *C. parvum* oocysts in water sample consists of the following five steps: (i) filtration of several tens of liters of sample through a 1.0-μm pore membrane, (ii) elution of retained particles by a buffered surfactant solution, (iii) centrifugal concentration of eluted particles, (iv) purification by immunomagnetic separation (IMS) with antibody-coated paramagnetic microspheres, (v) immunofluorescence assay (IFA) with fluorescein isothiocyanate-labeled monoclonal antibody (FITC-mAb) and 4′,6-diamidino-2-phenylindole (DAPI) staining followed by microscopic examination. Even though Method 1623, particularly the IMS step, greatly improved the purification efficiency of (oo)cysts compared to the previous Percoll-sucrose gradient method [5,6], the overall (oo)cyst recovery using Method 1623 still only averages approximately 50% [6-9].

In this study, we were interested in determining which steps in Method 1623 are responsible for the (oo)cyst loss, and to what extent, during this standard detection procedure. We therefore examined the (oo)cyst losses at each step, filtration, elution, centrifugal concentration, and IMS, by comparing the recovery yields of the following segmented analyses: (i) IMS + microscopic examination, (ii) centrifugal concentration + IMS + microscopic examination, and (iii) filtration/elution + centrifugal concentration + IMS + microscopic examination.

G. lamblia cysts were prepared by *in vitro* encystation of cultivated trophozoite cells, as described previously [10,11]. *C. parvum* oocysts were purchased from Waterborne, Inc. (New Orleans, LA, USA).

Our analyses were evaluated by following the proce-

***Corresponding author**
Tel: +82-31-330-6689 Fax: +82-31-336-6336
e-mail: kisay@mju.ac.kr

Table 1. Procedural steps for the detection of protozoan (oo)cysts

Step	Methods and Materials
(1) Filtration	PES capsule filter with 1-μm pore, 10 L spiked water
(2) Elution	Laureth-12™ elution buffer, wrist action shaker, eluted volume 500 mL
(3) Concentration	Centrifugal concentration to 5 mL
(4) Purification (IMS)	Antibody-coated paramagnetic microspheres, concentration to 50 μL
(5) IFA, DAPI staining, and microscopic examination	IFA with FITC-mAb, DAPI staining, epifluorescence microscope and differential interference contrast (DIC) examination

Table 2. Segmented examinations for the evaluation of (oo)cyst loss

	Spiked medium	Analysis procedure
Test 1	5 mL of IMS buffer	IMS + IFA microscopic examination
Test 2	500 mL of eluant buffer	Centrifugal concentration + IMS + IFA microscopic examination
Test 3	10 L of boiled tap water	Filtration/elution + centrifugal concentration + IMS + IFA microscopic examination

dure as directed in Method 1623 [4], the outline of which is summarized in Table 1. Water samples were prepared by spiking 10 L of boiled tap water with 160~200 carefully enumerated (oo)cysts. Boiling eliminates the chlorine from the tap water and also removes possible microbial contamination. The Envirocheck™ capsule filter (Pall Gelman Sciences, Ann Arbor, MI, USA), with a 1-μm absolute pore size pleated polyethersulfone (PES) membrane, was used for the filtration step at a rate of 2 L/min. For elution, the capsule filter was filled with 250 mL of Laureth-12™ elution buffer (PPG Industries, IL, USA) with PBS added, as recommended in Method 1623, and agitated with a wrist-action shaker (Pall Gelman). This step was performed twice. The eluant was then collected from the filter (approx. 500 mL) and centrifuged at 1,500 × g for 20 min to obtain the pellet fraction. The pellet volume obtained was always less than 1 mL in this study. The (oo)cysts were further purified from the pellet by IMS using the Dynabead™ GC-Combo IMS kit (Dynal, Inc., LA, USA), and the final volume was reduced to 50 μL. Purified samples were then transferred to multi-well slides, dried, and stained with the Aqua-Glo™ G/C Direct FL kit (Waterborne) and DAPI counterstained. An epifluorescence microscope was used for microscopic enumeration. Three replicate tests were completed, with an interval of longer than one week between the tests, and the results were averaged.

Recovery yields from spiked samples were examined in three segmented experiments (Table 2) in order to evaluate the loss in each step of (oo)cyst analysis. The number of (oo)cysts added to the samples ranged from 160 to 200, and the analyses were performed in triplicate. The sample for Test 1 simulated the concentrated pellet fraction (5 mL) obtained after centrifugal concentration of the eluant from the capsule filter. The sample for Test 2 simulated the eluant from the filter (500 mL). Test 3 was the complete Method 1623 procedure. As a result of these conditions, any (oo)cyst loss in Test 1 would be the result of the IMS step, assuming that (oo)cyst loss is negligible during microscopic examination. The difference in recovery yields between Test 1 and Test 2 would be (oo)cyst loss that occurred in the centrifugal concentration step, while the difference in recovery yields between Test 2 and Test 3 would be the combined loss from filtration and elution.

Table 3 shows the recovery percent in each test of this segmented analysis. The overall (oo)cyst recovery averaged 52~57%, when following the full procedure of Method 1623 (Test 3). In our lab, recovery yields are routinely in the range of 45.8~63.6% for *Cryptosporidium* oocysts and 40.8~71.3% for *Giardia* cysts, including this study, when using Method 1623.

Test 1, which started at the IMS step, exhibited very high recovery percents of 94~100% for both *Cryptosporidium* oocysts and *Giardia* cysts. Very little loss was observed for *Giardia* cysts, and only about 6% of *Cryptosporidium* oocysts were lost. Therefore, the current IMS technique is reliable for (oo)cyst purification and produces very little (oo)cyst loss, assuming that the lot-to-lot quality variation of the IMS kit is negligible and that the microscopic examination is accurate. It should be noted that the low rates of (oo)cyst loss in the IMS step may have been affected by the water used for spiking in this study. Here, we used relatively clean boiled tap water; the loss could be larger for real field samples with high turbidity.

Test 2 resulted in 85~87% of recovery. Because Test 2 began with the centrifugal concentration of the eluted solution, then proceeded with the same steps as Test 1, the difference in recovery percents between Test 1 and Test 2, about 8~14% of the spiked (oo)cysts, was the amount of (oo)cyst loss that occurred during the centrifugal concentration step. The (oo)cyst loss in this step accounted for 17~32% of the total (oo)cyst loss when the entire Method 1623 procedure is completed (Fig. 1). This step was designed to collect the (oo)cysts-containing sample and concentrate it to less than 5 mL through centrifugation from several hundred milliliters of

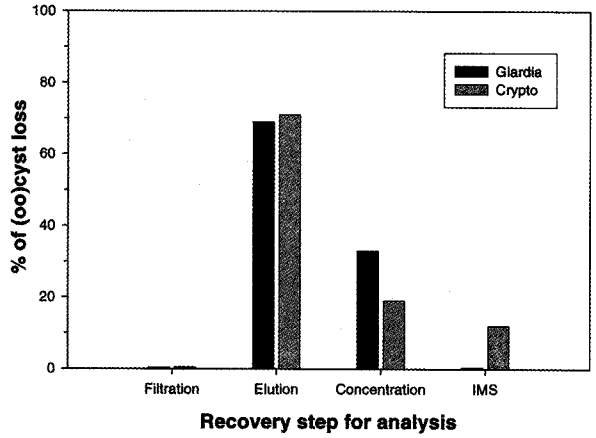

Fig. 1. Extent of (oo)cyst loss in each step of the analysis with respect to total loss.

eluted solution. The eluted solution underwent a series of volume reduction steps, including supernatant aspiration and transfer to a smaller tube for IMS, and the repeated handling seemed to responsible for the (oo)cyst loss.

Test 3 resulted in recovery of 52~57% of the (oo)cysts. Therefore, the difference in recovery percents between Test 2 and Test 3 ranged from 29~34% of the spiked (oo)cysts, and represents the loss due to the filtration/elution step. These values account for approximately 68~72% of the total loss, indicating that the majority of (oo)cyst loss takes place in the filtration/elution step, as shown in Fig. 1. In order to identify exactly which step, either filtration or elution, caused more of the (oo)cyst loss, we performed a separate analysis with 1 L of spiked water. By collecting and analyzing the 1-L permeate solution after filtration and careful centrifugal concentration, IMS, and a thorough microscopic examination, we observed that very few of (oo)cysts were found in the permeate solution (data not shown). Although we did not disintegrate the filter or examine the inside of the filter matrix, this result indicated that the permeated loss of (oo)cysts during filtration was negligible and that the filtering performance of the current PES capsule filter was satisfactory. Therefore, we conclude that the major cause of (oo)cyst loss in the filtration/elution steps, or in the analysis procedure as a whole, is poor elution of (oo)cysts from the filter matrix. Because we used the designated elution buffer and agitation method as directed in the Method 1623 protocol, we speculate that some physicochemical properties of the filter, such as the material itself or the pleated structure of the capsule filter, tend to retain the protozoan (oo)cysts too strongly. If so, the current elution buffer formulation or agitation conditions might not be optimal for the complete elution of (oo)cysts. As such, some improvements in the filtration and/or elution steps are necessary to enhance recovery efficiency in the current Method 1623 analysis system.

It should be noted that this study used boiled tap water as the water source that was spiked; this provided a relatively clean sample with low turbidity and contamination. Because this sample did not contain a large amount of

Table 3. Recovery yields in segmented evaluation tests

	Percent recovery (%) [mean ± S.D. (n = 3)]	
	Cryptosporidium oocyst	*Giardia* cyst
Test 1	94.1 ± 6.5	100.4 ± 8.8
Test 2	85.7 ± 8.2	86.4 ± 11.0
Test 3	52.3 ± 8.9	56.8 ± 15.2

suspended pellets, typically less than 1 mL per 10 L, the analysis steps after elution were much easier, and there was less interference. Therefore, the (oo)cyst loss obtained in this study would presumably be similar to that observed in the analyses of treated waters after filtration unit in water treatment plants (WTP) or of tap water at the locations of final users. However, (oo)cyst losses may increase in other steps of the procedure when analyzing field waters or samples from wastewater treatment facilities that contain a larger volume of pellets along with a higher population of parasitic protozoa. A higher volume of recovered pellets would interfere with the IMS reaction and microscopic examination, or could also give rise to an increase in false positives due to the increased microbial contamination.

Method 1623 from the US EPA is a widely accepted standard method for the detection and analysis of *C. parvum* oocysts and *G. lamblia* cysts in water. However, many researchers in this field have experienced insufficient recovery yields during the analysis, making it necessary to evaluate the (oo)cyst loss in each step involved in Method 1623 to identify which step(s) need improvements. The systematic review of Method 1623's steps demonstrated that the purification step employing IMS technique was fairly reliable and responsible only for 0~13% of the total (oo)cyst loss. The loss at the concentration step ranged from 17~32%, where centrifugation, aspiration, and sample transferring could be the causes of this loss. The majority of the (oo)cyst loss occurred during the elution step, which was responsible for 68~72% of the total loss. We suspect that the polymeric material itself or the pleated structure of the capsule filter used in the study might retain the protozoan (oo)cysts too strongly; thus, the current elution buffer formulation and the agitation conditions suggested in Method 1623 might not be optimal for the complete elution of (oo)cysts. Further improvements in the filtration/elution methodology are required to enhance the recovery yield and reliability in (oo)cyst analysis.

The efficiency of cyst elution from the filter membrane may be improved in several ways. First, the use of a disk-type membrane filter accompanied by kneading or scraping is an effective method for increasing cyst elution [12]. However, membrane filters usually have an increased tendency for fouling and a lowered filtration rate per unit time compared to capsule-type filters. Another possible remedy would be to change the composition of the elution buffer for a given filter material. The Laureth-12 elution buffer used in this study for the PES capsule filter is a PBS-based elution buffer supplemented with a sur-

factant mixture of SDS and Tween 80. According to our previous experiments that examined the effects of changing the surfactant composition and pH on elution [13], Tween 20 was better than Tween 80 for cyst elution. The Tween series of surfactants are nonionic and share a common sorbitan ring with different numbers of polyoxyethylene units. Tween 20 has a higher HLB (hydrophilic-lipophilic balance) value than Tween 80 does. An alkaline pH between 8 and 10 was also better for elution conditions from the PES capsule filter compared to an acidic or neutral pH range.

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