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Modifications to United States Environmental Protection Agency Methods 1622 and 1623 for Detection of *Cryptosporidium* Oocysts and *Giardia* Cysts in Water

Randi M. McCuin and Jennifer L. Clancy*

Clancy Environmental Consultants, Inc., St. Albans, Vermont 05478

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Collaborative and in-house laboratory trials were conducted to evaluate *Cryptosporidium* oocyst and *Giardia* cyst recoveries from source and finished-water samples by utilizing the Filta-Max system and U.S. Environmental Protection Agency (EPA) methods 1622 and 1623. Collaborative trials with the Filta-Max system were conducted in accordance with manufacturer protocols for sample collection and processing. The mean oocyst recovery from seeded, filtered tap water was 48.4% ± 11.8%, while the mean cyst recovery was 57.1% ± 10.9%. Recovery percentages from raw source water samples ranged from 19.5 to 54.5% for oocysts and from 46.7 to 70.0% for cysts. When modifications were made in the elution and concentration steps to streamline the Filta-Max procedure, the mean percentages of recovery from filtered tap water were 40.2% ± 16.3% for oocysts and 49.4% ± 12.3% for cysts by the modified procedures, while matrix spike oocyst recovery percentages ranged from 2.1 to 36.5% and cyst recovery percentages ranged from 22.7 to 68.3%. Blinded matrix spike samples were analyzed quarterly as part of voluntary participation in the U.S. EPA protozoan performance evaluation program. A total of 15 blind samples were analyzed by using the Filta-Max system. The mean oocyst recovery percentages was 50.2% ± 13.8%, while the mean cyst recovery percentages was 41.2% ± 9.9%. As part of the quality assurance objectives of methods 1622 and 1623, reagent water samples were seeded with a predetermined number of *Cryptosporidium* oocysts and *Giardia* cysts. Mean recovery percentages of 45.4% ± 11.1% and 61.3% ± 3.8% were obtained for *Cryptosporidium* oocysts and *Giardia* cysts, respectively. These studies demonstrated that the Filta-Max system meets the acceptance criteria described in U.S. EPA methods 1622 and 1623.

Prior to 1998, the method recommended by the U.S. Environmental Protection Agency (EPA) for protozoan analysis of drinking water in the United States was the information collection rule (ICR) method (20). Problems with the ICR method are well documented and include low recovery efficiencies, high false-positivity and false-negativity rates, and poor precision and accuracy. The method is labor intensive, and recoveries are erratic, even in expert laboratories (7). In the United Kingdom, the Standing Committee of Analysts (SCA) developed an approach similar to the ICR method (1). Following the dramatic outbreaks of waterborne cryptosporidiosis in the 1990s, it was widely accepted that there was a need to develop an improved method for detecting protozoan pathogens in water. In 1997, the U.S. EPA developed a new method for analysis of protozoa in water, U.S. EPA method 1622 for *Cryptosporidium* detection in water by filtration, immunomagnetic separation (IMS), and fluorescent-antibody staining (FA) (22). Method performance was shown to be significantly improved (9, 24), with the following improvements: (i) a new filter design for increased efficiency of oocyst capture and elution; (ii) incorporation of IMS, permitting concentration of specific microorganisms to reduce false positives and nonspecific interferences; (iii) an additional staining step to further aid in confirmed identification of (oo)cysts; and (iv) stringent quality assurance (QA) and quality control (QC) measures. Following the commercial production of IMS beads for the specific capture of *Giardia* cysts, method 1623 for *Giardia* and *Cryptosporidium* detection in water by filtration, IMS, and FA was developed (23) and validated in collaborative trials (25). Original validation studies for each method used the Pall Gelman Envirochek capsule filter, but since that time, three other filters have been validated for use in methods 1622 and 1623: the Whatman CrypTest cartridge filter, the Pall Gelman Envirochek HV capsule for *Cryptosporidium*, and the IDEXX Filta-Max system. Final versions of methods 1622 (26) and 1623 (27) are available on the U.S. EPA website at www.epa.gov.

The IDEXX Filta-Max system was originally introduced as an improvement to the SCA method in the United Kingdom to concentrate and recover *Cryptosporidium* in both tap and source water samples (19). Direct examination of filter eluates from seeded tap water (volumes ranging from 100 to 2,000 liters) resulted in oocyst recovery percentages ranging from 76.7 to 106.7% (1×10^3 to 3.1×10^3 oocysts seeded). With source water samples (10- to 20-liter samples), a mean oocyst recovery percentage of 88.8% ± 5.9% was obtained (3×10^3 oocysts seeded). When filter eluate subsamples were subjected to a single density gradient flotation step, the mean oocyst recovery percentage declined to 32.8% ± 13.5% for finished-water samples and 24% ± 6.9% for source water samples.

The poor recovery efficiencies associated with these methods, all of which relied upon flotation as a processing step (4,

* Corresponding author. Mailing address: Clancy Environmental Consultants, Inc., P.O. Box 314, St. Albans, VT 05478. Phone: (802) 527-2460. Fax: (802) 524-3909. E-mail: jclancy@clancyenv.com.

12), suggest that the concentration step of these methods required improvement. A number of studies have reported IMS to be a superior alternative to density gradient flotation techniques for isolating *Cryptosporidium* oocysts and *Giardia* cysts from environmental water matrices (5, 6, 13, 16, 18; R. M. McCuin, T. M. Hargy, J. E. Amburgey, and J. L. Clancy, Proc. Am. Water Works Assoc. Water Quality Technical Conference, abstr. T35, 2001). Separate studies demonstrated that IMS yields consistently higher oocyst and cyst recovery percentages with lower variability than flotation procedures. In three matrices tested, Hsu and Huang (13) showed a mean oocyst recovery percentage of 69.3% ± 13.3% with IMS, compared to 29.9% ± 20.3% with Percoll-sucrose flotation. The mean cyst recovery percentage was 78.6% ± 13.8% with IMS, while only 22.7% ± 14.0% recovery efficiency was achieved by using Percoll-sucrose flotation with these same matrices. In round robin trials conducted by Campbell and Smith (6), the mean IMS oocyst recovery percentage was significantly higher, at 74 0.1% ± 12%, than that achieved by the SCA method (57.8% ± 18.8%). When the turbidity of the sample was increased to 40 to 60 nephelometric turbidity units (ntu), the mean oocyst recovery percentages achieved by the IMS and SCA methods were similar, at 46.3% ± 10.2% and 47.6% ± 10.4%, respectively. The percentage of oocyst recovery from 600 ntu of water achieved by the SCA method was similar (43.8% ± 25%); however, the IMS recovery percentage dropped significantly to 29.0% ± 34.0%. In contrast, Bukhari et al. (5) and McCuin et al. (16) observed comparable (oo)cyst recovery percentages when IMS was used across a wide range of turbidities (50 to 5,000 ntu).

In this communication, we describe the results of interlaboratory trials of the Filta-Max system for the capture and recovery of *Cryptosporidium* oocysts and *Giardia* cysts from water samples seeded with low concentrations of microorganisms. The study design was that prescribed by the U.S. EPA in the performance-based measurement system (PBMS) approach to method improvement (21). Following concentration, recovered (oo)cysts were isolated from the interfering debris by using IMS and detected by using fluorescent monoclonal antibodies (MAbs). The laboratories involved in the collaborative trials were all certified for water testing for protozoan parasites by the methods described in the U.S. EPA ICR (20) and currently participate in the U.S. EPA protozoan performance evaluation (PE) program. To avoid the need to sanitize the Filta-Max wash station and its elution accessories, modified sanitation procedures and the employment of disposable alternatives to the Filta-Max wash station and particle concentrator were also investigated in the present study. The wash station was replaced with a stomaching method to recover (oo)cysts from the filter material. The particle concentrator was replaced with centrifugation of the eluate for sample concentration prior to IMS. These modifications were collaboratively tested in four laboratories with filtered tap water, as well as source water matrices. In addition, the Filta-Max system was evaluated for recovery of (oo)cysts from finished-water samples at various (oo)cyst concentrations.

MATERIALS AND METHODS

Stock suspension preparation and enumeration. The *Cryptosporidium parvum* oocysts used in this study were originally obtained from Harley Moon (National Animal Disease Center, Ames, Iowa). The Iowa strain is maintained and propagated at the Sterling Parasitology Laboratory (Department of Veterinary Science, University of Arizona, Tucson) as described previously (14). Briefly, the total daily fecal output of infected calves was collected, screened through sieves, and concentrated by centrifugation. Oocysts were isolated from the feces by using discontinuous sucrose gradients and then microcentrifuge scale cesium chloride gradients (2, 3). Purified oocysts were stored at 4°C in 0.01% Tween 20 solution containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 100 μg of gentamicin per ml of oocyst suspension to retard bacterial growth. These oocysts were used for no more than 12 weeks following shedding and purification. Stock suspensions were diluted to 10^6 oocysts ml^{-1} with deionized water (DI), and oocysts were enumerated with a hemocytometer (Hausser Scientific, Horsham, Pa.) as described in method 1623 (27).

Giardia intestinalis cysts were obtained from Waterborne, Inc. (New Orleans, La.). The H3 strain of *G. intestinalis* cysts was originally isolated from a human and has been maintained by passage through Mongolian gerbils. Cysts were purified by sucrose density gradient centrifugation, followed by Percoll gradient purification. Purified cysts were stored in DI and antibiotics at 4°C. Cysts used for this study were less than 30 days old. Stock suspensions of cysts were diluted to 10^6 ml^{-1} with DI, and cysts were enumerated with a hemocytometer (27).

Preparation and enumeration of working spike dose suspensions of (oo)cysts.
(i) Manual enumeration. Spike dose suspensions were prepared and enumerated as outlined in methods 1622 (26) and 1623 (27). Briefly, individual working suspensions of *C. parvum* and *G. intestinalis* were prepared by diluting an aliquot of the stock suspension in DI to approximately 10^3 (oo)cysts ml^{-1}. Ten 100-μl aliquots were placed into individual wells of three-well treated microscope slides (Meridian Diagnostics, Cincinnati, Ohio) and stained as described below. Spike dose suspensions were used within 24 h of enumeration.

(ii) Flow cytometry-counted suspensions. Spike dose suspensions were obtained from the Wisconsin State Laboratory of Hygiene (WSLH) in Madison. These suspensions were prepared by flow cytometry in accordance with protocols used by the U.S. EPA in its PE studies (11, 24, 25). The WSLH obtained fresh oocysts from the Sterling Parasitology Laboratory (University of Arizona, Tucson) and cysts from Waterborne, Inc. Spikes were prepared with an Epics Elite flow cytometer equipped with a cell sorter (Coulter Corp., Miami, Fla.). The flow cytometer was set up in accordance with manufacturer directions and calibrated by using precisely enumerated suspensions of control beads to ensure proper instrument performance. Parasite suspensions were then sorted on the basis of their forward light scatter properties to determine size and their side light scatter properties to determine internal complexity. A sort region based upon white light measurements was drawn around the parasites, and the instrument was programmed to distribute the target number of parasites onto three-well microscope slides. Instrument calibration was performed by sorting 10 replicate wells (per organism) at the target level and enumerating these preparations by epifluorescence microscopy at a magnification of ×200. Cytometer accuracy and reproducibility were determined by calculating the mean and standard deviation of the replicate wells. Instrument calibration was performed immediately before spike preparation. During spike preparation, the slide holder was replaced with a spike collection tube containing reagent grade water (18 MΩ-cm resistivity) with 0.01% Tween 20 and cysts or oocysts were sorted directly into the tube. After each set of 10 spikes, an ongoing calibration check was performed as described above and the organism count was verified microscopically. Suspensions were sent to laboratories by overnight courier. Each laboratory received conical tubes containing an unknown number of (oo)cysts. A single vial was used for each test. All spike dose suspensions were used within 24 h of receipt.

Source water trials with 50-liter sample volumes and the Filta-Max system. In accordance with the PBMS study design, a total of eight samples per laboratory were spiked with blinded flow cytometry sorted vials of (oo)cysts and processed in four laboratories (five reagent water samples [initial precision and recovery or IPR] and three matrix spike [MS] samples per laboratory). Spiking was accomplished by adding the contents of a single conical vial to a 10-liter sample in a carboy. The vial was rinsed with phosphate-buffered saline (PBS) containing 0.01% Tween 20 (PBST) three times, and the rinses were added to the respective carboys. The samples were filtered through Filta-Max filters at a flow rate of 2 liters min^{-1} with the aid of a peristaltic pump located on the inlet side of the filter. When 9 liters of the sample had been filtered, a second 10-liter volume of reagent water or source water was added to the carboy. Subsample additions were repeated until a total volume of 50 liters had passed through each test filter.

Filters were washed in accordance with the protocol prescribed by the manufacturer. Briefly, this involved placing a membrane filter (3-μm pore size; polysulfone) in the base of the sample concentrator, inserting the filter module into the apparatus, adding 600 ml of PBST to the reservoir, and unscrewing the filter housing to allow expansion of the foam pads. Foam pads were washed by

pumping the plunger and then transferring the PBST elution volume into the magnetic particle concentrator and filtering the entire volume to approximately 20 to 25 ml under vacuum. The process was repeated with a second 600-ml volume of PBST that was pooled with the first concentrate and then filtered under vacuum to produce a final volume of around 20 ml. The concentrate was then transferred into a 50-ml centrifuge tube, the filter membrane was transferred to a small plastic Zip-loc bag, and 8 to 10 ml of PBST was added. The membrane was kneaded manually, and the spent wash volume was pooled with the primary eluate. The membrane washing procedure was repeated twice. The sample was resuspended to 50 ml with PBST and subjected to centrifugation at 2,000 × *g* for 10 min. The supernatant was aspirated to 10 ml, and the resuspended pellet was transferred to a Leighton tube and subjected to IMS.

Dynal IMS procedure for *Giardia* cysts and *Cryptosporidium* oocysts. The IMS procedure was performed as described in U.S. EPA method 1623 (27). Briefly, each 10-ml sample concentrate was added to a Leighton tube containing 1 ml of 10× SL buffer A and 1 ml of 10× SL buffer B (Dynal, A.S., Oslo, Norway). One hundred microliters of *Cryptosporidium* and *Giardia* IMS beads was added to each tube, and samples were incubated for 1 h at room temperature with constant rotation. The Leighton tubes were then placed in a magnetic particle concentrator and gently rocked for 2 min through a 90° angle. The supernatant was decanted, the tubes were removed from the magnetic particle concentrator, and 1 ml of 1× SL buffer A was added to each tube. The tubes were gently rocked to resuspend the bead-(oo)cyst complexes, a Pasteur pipette was used to transfer the suspension into a 1.5-ml polypropylene tube, and the tubes were placed in a second magnetic particle concentrator and rocked for 1 min. Supernatants were aspirated, the magnet was removed, and 50 μl of 0.1 N HCl was added to each sample, which was vortexed for a minimum of 10 s. Samples were allowed to stand for 10 min in the upright position and vortexed for a further 10 s, and magnets were inserted. The tubes were allowed to stand undisturbed for 2 min. The resulting supernatant was placed in the center of a well of a three-well microscope slide containing 5 μl of 1 N NaOH.

Staining procedure for recovered (oo)cysts. The samples were dried (42°C for 1 to 2 h), fixed in methanol, and air dried, and 50 μl of fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* sp. and anti-*Giardia* sp. MAbs (Waterborne Inc.) was placed onto each well. The slides were placed in a humidified chamber and incubated (37°C for 30 min), and excess FITC-MAb was aspirated. Any remaining FITC-MAb was removed by adding 50 μl of PBS to each well, allowing the slides to stand for 1 min, and aspirating the excess PBS. A 50-μl aliquot of 4′,6′-diamidino-2-phenylindole (DAPI) solution (0.4 μg ml^{-1} in PBS) was introduced into each well. The slides were allowed to stand at room temperature for 2 min, and excess DAPI solution was removed by washing the slides twice in PBS and once in DI. The slides were placed in the dark until dry, a 10-μl aliquot of mounting medium (2% diazabicyclooctane [DABCO] in 60% glycerol–40% PBS) was introduced onto each well, and slides were sealed for subsequent examination under epifluorescence optics as described in method 1623 (27).

Trials to determine if disinfection and cleaning procedures were adequate to reduce carryover contamination. A carboy containing 10 liters of filtered (1-μm pore size) tap water was seeded with high levels of *C. parvum* oocysts and *Giardia intestinalis* cysts (concentrations ranged from 2×10^4 to 1×10^6/liter). The seeded volume was passed through a Filta-Max filter in accordance with the manufacturer's recommendations; this was followed by elution of the (oo)cysts and concentration with the wash station and particle concentrator. Following IMS concentration, samples were examined by epifluorescence microscopy. After samples were eluted from the filters, the spiking carboy, filter housing, and wash station components were disinfected and cleaned as described below. The same components (carboy, filter housing, and wash station) were then used to process an unspiked negative control. The spiking and negative control trials were performed three times, and between sample tests, the disinfection-and-cleaning protocol described above was employed.

All parts of the Filta-Max housing, wash station, and particle concentrator and all parts of the carboy-spiking setup were disassembled. O rings were removed from the wash station and filter housing. All parts were thoroughly rinsed with tap water and immersed in a 6% sodium hypochlorite solution. After 30 min, each part was removed from the hypochlorite bath; rinsed with tap water; scrubbed vigorously with hot, soapy tap water; and then subjected to multiple rinses with tap water and DI. The plunger from the wash station was cleaned by securing a 600-ml beaker containing 300 ml of 6% hypochlorite to the jaws of the wash station base. The plunger was pulled down until it was immersed in the hypochlorite. The plunger head was allowed to stand for 30 min at room temperature and then carefully removed and immersed in a 600-ml beaker containing 300 ml of tap water. The plunger was rinsed by being moved up and down five times. With a scrub pad, the plunger head was scrubbed with hot, soapy water and rinsed five times in a 600-ml beaker containing 300 ml of tap water. The plunger head was given a final rinse with DI and dried with paper toweling.

Use of a stomacher and centrifugation as a replacement for the Filta-Max wash station and particle concentrator. In accordance with the PBMS study format, eight samples were spiked in each of four laboratories as described previously. The content of a single conical vial was added to a 50-liter sample in a carboy. The vial was rinsed three times with PBST, and the rinses were added to the respective carboys. The samples were filtered through Filta-Max filters at a flow rate of 2 liters min^{-1} with the aid of a peristaltic pump located on the inlet side of the filter. Retained (oo)cysts were eluted from the filters by replacing the wash station with a stomacher (Seward Medical, London, England). Concentration of the filter eluate was accomplished by centrifugation instead of using the particle concentrator. Briefly, this involved placing the filter in a large stomacher bag. With the Allen key provided by the manufacturer, the bolt was removed from the center of the filter, allowing the pads to expand within the stomacher bag. The bolt and plastic ends were rinsed with PBST and discarded. A 600-ml volume of PBST was added to the stomacher bag, and the bag was stomached for 5 min at normal speed. The wash solution was decanted into a 2-liter pooling beaker, a second 600-ml volume of PBST was added to the stomacher bag, and the washing step was repeated. After the second wash, the pads in the bag were removed and the retained liquid was expressed from the pads by squeezing them over the pooled filtrate. Residual solution from the spent bag was also decanted and added to the pooled filtrate. The stomacher bag was rinsed with PBST, and this rinse volume was added to the pooled solution. The pooled filtrates were then transferred to 250-ml conical centrifuge tubes and centrifuged at 1,500 × *g* for 15 min. The supernatant was aspirated to approximately 5 ml above the packed pellet, and the pellets from each bottle were combined into a 50-ml centrifuge tube and further concentrated by centrifugation (1,500 × *g* for 15 min). The supernatant was aspirated to 10 ml, and the resuspended pellet was transferred to a Leighton tube for IMS.

Demonstration of precision and accuracy with reagent water and blinded PE samples. Blinded PE samples were received quarterly from the U.S. EPA as part of its continuing program for laboratories seeking to demonstrate QA through an independent laboratory testing scheme. The PE vials were prepared by flow cytometry at the WSLH and shipped by overnight courier to the testing laboratory. Each blind spike dose vial contained either *Cryptosporidium* oocysts, *Cryptosporidium* oocysts and *Giardia* cysts, or no organisms. Also contained in each vial was a reference matrix (Tennessee River sediment). The sample volumes analyzed ranged from 10 liters (rounds 1 and 2) to 50 liters (rounds 3 through 5). In rounds 1 through 3, each sample was processed with the wash station and particle concentrator for elution and concentration. Recovered organisms were isolated from interfering debris by IMS. In rounds 4 and 5, the wash station and particle concentrator were replaced with the stomaching-centrifugation modification for sample concentration. With each round, in-house positive and negative control samples were analyzed in the same manner as the blinded PE samples. These controls were performed with filtered tap water (10 or 50 liters). Positive control samples were seeded with *Cryptosporidium* oocysts and *Giardia* cysts, which were enumerated manually in accordance with the protocols outlined in methods 1622 (26) and 1623 (27).

Evaluation of the Filta-Max system for recovery of (oo)cysts from finished water. Following filtration of 10-liter volumes of prefiltered tap water (flow rate, 2 liters min^{-1}) through the Filta-Max filter, *C. parvum* oocysts and *G. intestinalis* cysts were spiked directly into the filter housing. An additional 990 liters of prefiltered tap water was then passed through each filter. (Oo)cysts were eluted from the filter material with the wash station. For spike doses of >100 organisms, subsamples (20%) of each filter eluate were collected on 25-mm-diameter, 1.0-μm-pore-size polycarbonate track etch (PCTE) filters as described below. Recovered (oo)cysts were enumerated by epifluorescence microscopy. The remaining eluate from each filter was concentrated with the particle concentrator as previously described. Recovered (oo)cysts were isolated from the concentrate with the Dynal GC combo IMS kit. When filters were spiked with 50 or 100 (oo)cysts, the entire sample volume was concentrated with the particle concentrator and subjected to IMS.

Detection of (oo)cysts on 25-mm-diameter membrane filters. A 25-mm-diameter, 1.0-μm-pore-size PCTE membrane filter (Whatman) was placed on a glass chimney filtration assembly (Millipore, Inc., Bedford, Mass.), and 20% of the filter eluate was filtered under vacuum (15 lb/in^2). The inlet side of the glass chimney was rinsed twice with 0.01% Tween 20 (5-ml wash followed by a 10-ml wash). For each filter, a humidified chamber was constructed by placing a wet paper towel inside a plastic petri dish (100 by 15 mm). A smaller petri dish cover (60 by 15 mm) was inverted, and 100 μl of combined *Cryptosporidium-Giardia* FITC-MAb was blotted onto the surface. The PCTE membrane filter was placed on top of the FITC-MAb droplet, the large petri dish cover was applied, and the

TABLE 1. *Cryptosporidium* and *Giardia* data from the collaborative trial of method 1623 with the Filta-Max system and 50-liter source water samples

Sample type	Laboratory no.	Turbidity (ntu)	Spike dose (no. of [oo]cysts)		% Recovery		Mean % recovery[b]		RSD[c]/RPD[d]	
			Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
IPR 1	1[a]	<0.05	101	100	50.6	51.2				
IPR 2	1	<0.05	101	100	48.6	54.2				
IPR 3	1	<0.05	101	100	44.6	46.1				
IPR 4	1	<0.05	101	100	57.5	68.2	50.3	54.9	10.7	17.2
MS 1	1	1.0	101	100	54.5	63.2				
MS 2	1	1.0	101	100	42.6	60.2	48.6	61.7	24.5	4.8
IPR 1	2	<0.05	103	103	62.4	70.0				
IPR 2	2	<0.05	103	103	49.7	70.0				
IPR 3	2	<0.05	103	103	41.9	58.4				
IPR 4	2	<0.05	103	103	45.8	67.1	50.0	66.4	17.8	8.3
MS 1	2	4.8	103	103	23.4	69.1				
MS 2	2	4.8	103	103	19.5	57.4	21.4	63.2	18.2	18.5
IPR 1	3	<0.05	103	103	53.6	58.4				
IPR 2	3	<0.05	103	103	49.7	52.5				
IPR 3	3	<0.05	103	103	53.6	40.9				
IPR 4	3	<0.05	103	103	48.7	54.5	51.4	51.6	5.0	14.6
MS 1	3	6.3	103	103	48.7	55.4				
MS 2	3	7.2	103	103	49.7	54.5	49.2	55.0	2.0	1.8
IPR 1	4	<0.05	103	103	48.7	63.2				
IPR 2	4	<0.05	103	103	23.4	10.0				
IPR 3	4	<0.05	103	103	25.3	33.1				
IPR 4	4	<0.05	103	103	71.2	56.4	42.2	55.7	53.4	28.9
MS 1	4	15.6	103	103	39.0	69.1				
MS 2	4	15.6	103	103	48.7	45.7	43.9	57.4	22.2	40.7

[a] One *Giardia* cyst was observed in the unspiked reagent water sample.
[b] Range of IPR recoveries to meet acceptance criteria (Method 1623) for *Cryptosporidium* and *Giardia* 21 to 100% and 17 to 100%, respectively. Range of recoveries in matrix samples must fall between 13 and 111% for *Cryptosporidium* and between 15 and 118% for *Giardia*.
[c] Relative standard deviation (IPR only) − RSD values for *Cryptosporidium* and *Giardia* must fall below 40% and 41%, respectively, to meet acceptance criteria for Method 1623.
[d] Relative percent difference (MS only) − RPD values for *Cryptosporidium* and *Giardia* must fall below 61% and 30%, respectively, to meet acceptance criteria for Method 1623.

sample was incubated at 35°C for 60 min. Each filter was removed from the humidified chamber and placed on the glass support of the filtration assembly, and excess FITC-MAb was removed under gentle vacuum. With the vacuum on, approximately 1 ml of PBS was pipetted gently onto the filter surface in order to rinse off excess FITC-MAb. The filter was then transferred onto a slide (25 by 75 mm) containing 50 μl of DABCO mounting medium. Another 25 μl of DABCO was placed on top of the filter, a coverslip was applied, and the filter was sealed with clear nail polish and examined by epifluorescence microscopy to quantify the (oo)cysts.

Microscopic examination. A Zeiss Axioskop fluorescence microscope equipped with a blue filter block (excitation wavelength, 490 nm; emission wavelength, 510 nm) was used for detection of FITC-MAb-labeled oocysts at a magnification of ×320. Confirmation of oocysts was achieved at a magnification of ×640 by using a UV filter block (excitation wavelength, 400 nm; emission wavelength, 420 nm) for visualization of DAPI-stained nuclei, and the internal morphology of (oo)cysts was confirmed under Nomarski-differential interference contrast microscopy.

RESULTS

Collaborative trials with 50-liter source water samples. For *Giardia* and *Cryptosporidium,* the mean recovery percentages and relative standard deviations (RSD) of the four filtered tap water sample tests were calculated for each laboratory and test organism. Table 1 shows that (oo)cyst recovery efficiency percentages for filtered tap water ranged from 23.45 to 71.2% for *Cryptosporidium* with a mean recovery percentage of 48.5% ± 11.8% for the four participating laboratories. *Cryptosporidium* RSD ranged from 5.0% to 53.4%. The mean percentage of efficiency of *Giardia* recovery from filtered tap water (57.1% ± 11.0%) was marginally higher and somewhat less variable than the oocyst recovery data. The cyst recovery percentages from filtered tap water ranged from 33.1 to 70.0%. RSD for *Giardia* recovery percentages ranged from 1.8 to 28.9% in the four laboratories. One laboratory detected a single *Giardia* cyst in the unseeded water blank. The opportunity for carryover contamination when working with *Giardia* and *Cryptosporidium* is high, as (oo)cysts are sticky and difficult to remove from labware by washing. The use of unspiked blank samples is important to assess laboratory hygiene. Subsequent investigation in this laboratory showed that additional unspiked blank samples were negative, and no obvious contamination of labware, reagents, etc., was found.

Table 1 shows MS recovery data. Turbidity levels of each matrix used were determined at the individual laboratories. Relative percent difference (RPD) was calculated for each organism in each laboratory. MS *Cryptosporidium* recovery percentages ranged from 19.5 to 54.5%, while *Giardia* recovery percentages ranged from 45.7 to 70.0%. Three oocysts and 25 cysts were observed in the unspiked matrix sample analyzed by one of the testing laboratories (laboratory 1). The background levels of (oo)-

TABLE 2. *Cryptosporidium* and *Giardia* data from the collaborative trial of method 1623 with the stomacher and centrifugation for sample processing

Sample type	Laboratory no.	Turbidity (ntu)	Spike dose (no. of [oo]cytes)		% Recovery		Mean % recovery[a]		RSD[b]/RPD[c]	
			Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
IPR 1	1	<0.05	95	95	45.3	67.3				
IPR 2	1	<0.05	95	95	40.0	56.3				
IPR 3	1	<0.05	95	95	41.0	58.3				
IPR 4	1	<0.05	95	95	44.2	56.3	42.6	59.6	5.9	8.9
MS 1	1	4.6	95	95	30.5	68.3				
MS 2	1	4.3	95	95	32.6	58.3	31.6	63.3	6.7	15.9
IPR 1	2	<0.05	101	101	68.1	40.5				
IPR 2	2	<0.05	101	101	55.3	58.3				
IPR 3	2	<0.05	101	101	70.1	42.5				
IPR 4	2	<0.05	101	101	51.3	61.3	61.2	50.6	15.2	21.0
MS 1	2	2.5	101	101	36.5	22.7				
MS 2	2	2.6	101	101	35.5	26.7	36.0	24.7	2.7	16.0
IPR 1	3	<0.05	146	148	47.4	58.1				
IPR 2	3	<0.05	146	148	37.8	67.5				
IPR 3	3	<0.05	146	148	48.7	50.6				
IPR 4	3	<0.05	146	148	35.7	64.8	42.4	60.3	15.6	12.5
MS 1	3	4.9	146	148	17.2	54.7				
MS 2	3	4.9	146	148	16.5	67.5	16.8	61.1	4.1	21.0
IPR 1	4	<0.05	146	148	19.2	34.4				
IPR 2	4	<0.05	146	148	15.1	32.4				
IPR 3	4	<0.05	146	148	10.3	29.0				
IPR 4	4	<0.05	146	148	16.5	43.9	15.3	34.9	24.4	18.2
MS 1	4	1.29	146	148	12.4	30.4				
MS 2	4	1.29	146	148	12.4	38.5	12.4	34.4	0	23.5

[a] Range of IPR recoveries to meet acceptance criteria (Method 1623) for *Cryptosporidium* and *Giardia* 21 to 100% and 17 to 100%, respectively. Range of recoveries in matrix samples must fall between 13 and 111% for *Cryptosporidium* and between 15 and 118% for *Giardia*.

[b] Relative standard deviation (IPR only) − RSD values for *Cryptosporidium* and *Giardia* must fall below 40% and 41%, respectively, to meet acceptance criteria for Method 1623.

[c] Relative percent difference (MS only) − RPD values for *Cryptosporidium* and *Giardia* must fall below 61% and 30%, respectively, to meet acceptance criteria for Method 1623.

cysts were subtracted from the total number detected in each seeded matrix sample as recommended in method 1623. (Oo)cysts were not detected in the unspiked matrix samples analyzed at the other laboratories. Data from all four laboratories met the method acceptance criteria developed through collaborative testing conducted by the U.S. EPA for initial validation of the methods. The U.S. EPA has approved the Filta-Max system for use in all laboratories for methods 1622 and 1623.

Disinfection and sanitation trial results. Three disinfection and cleaning trials were conducted by spiking samples with very high (oo)cyst numbers (10^4 to 10^5), levels that are unlikely to be encountered in environmental source water samples. With such extreme seeding levels, carryover contamination can become problematic. However, in the three trials conducted, after rigorous disinfection and cleaning protocols were employed, no (oo)cysts were detected in the negative control samples, indicating that the sanitation process employed was effective in removing (oo)cysts from the equipment (data not shown).

Modifications of the Filta-Max elution and concentration techniques for (oo)cyst recovery from source water. Results of collaborative trials of (oo)cyst recovery from source water with the modified filter elution and sample concentration techniques are presented in Table 2. For *Cryptosporidium* and *Giardia,* the mean recovery percentages and RSD from the filtered tap water samples were calculated for each laboratory. When the modified method was used, oocyst recovery percentages ranged from 10.3 to 70.1%, with a mean recovery of 40.4% ± 17.8%. Mean oocyst recovery percentages observed within each laboratory ranged from 15.3 to 61.2%. RSD ranged from 5.9 to 24.4%. The mean *Giardia* cyst recovery from filtered tap water was 51.4% ± 12.6%, with cyst recovery percentages ranging from 29.0 to 67.5%. The mean cyst recovery percentages observed within each laboratory ranged from 34.9 to 60.3%, with RSD ranging from 8.9 to 21.0%. Recovery of *Cryptosporidium* oocysts from matrix samples ranged from 12.4 to 36.5%, while *Giardia* cyst recovery percentages ranged from 22.7 to 68.3%. *Cryptosporidium* RPD values ranged from 0 to 6.7%. *Giardia* RPD values were slightly higher and ranged from 15.9 to 21.0%.

The analytic data generated in this study met the 1623 method acceptance criteria developed through collaborative testing conducted by the U.S. EPA for initial validation of the method, with the exception of the *Cryptosporidium* IPR and MS data from a single laboratory (laboratory 4). In this situation, the three laboratories that met the method acceptance criteria can elect to use the modification for future sample processing. However, laboratory 4 would need to demonstrate its competency with the stomacher-centrifugation technique by

TABLE 3. *Cryptosporidium* and *Giardia* recovery data for blinded PE samples and associated QA and QC samples

Sample type	Elution/concn method[a] (sample vol [liters])	*Giardia* spike dose (no. of cysts)	% Recovery	*Cryptosporidium* spike dose (no. of oocysts)	% Recovery
QA 1	WS/PC (10)	544 ± 27	63	546 ± 22	51
PE 1		100	34	100	36
PE 2		200	56	200	47
PE 3		0	0	0	0
QA 2	WS/PC (10)	83 ± 10	74	127 ± 13	42
PE 4		NR[b]	NR	75	33
PE 5		NR	NR	125	37
PE 6		NR	NR	0	0
QA 3	WS/PC (50)	117 ± 15	74	100 ± 15	59
PE 7		100	54	100	50
PE 8		100	38	100	48
PE 9		0	0	0	0
QA 4	S/C (50)	106 ± 22	42	124 ± 15	29
PE 10		125	27[c]	125	69[c]
PE 11		125	47[c]	125	69[c]
PE 12		0	0	0	0
QA 5	S/C (50)	99	54	99	45
PE 13				100	64
PE 14				125	40
PE 15				0	0

[a] WS/PC, wash station-particle concentrator method; S/C, stomacher-centrifugation method.
[b] NR, results not reported by U.S. EPA because of poor staining of *Giardia* cysts.
[c] Analyst noted that cysts were poorly stained.

repeating the eight-sample trial successfully before adopting the technique for routine sample processing.

Precision and accuracy with filtered tap water and blinded PE samples. Results obtained with QA-QC samples and blinded PE samples are presented in Table 3. Recovery of oocysts from QA samples (filtered tap water) ranged from 29.1 to 59.2%, with a mean of 45.5% ± 11.2% with spike doses of approximately 100 organisms, with the exception of the first QA sample with a spike dose of ~500 oocysts. Cyst recovery percentages were slightly higher, ranging from 41.6 to 74.4%, with a mean cyst recovery of 61.3% ± 13.7%. The mean oocyst recovery from PE filtered tap water samples was slightly higher, at 50.2% ± 13.7%, with recovery percentages ranging from 33.3 to 68.8%. Recovery of *Giardia* cysts from the PE matrix declined somewhat (41.2% ± 9.9%) compared to recovery from filtered tap water (61.3% ± 13.7%). No decline in recovery was noted when sample volumes were increased from 10 to 50 liters. When the elution-sample concentration technique was changed from the Filta-Max wash station and particle concentrator to stomacher-centrifugation, the mean oocyst recovery from the QA-QC samples declined from 51.0 to 37.2%. However, the mean oocyst recovery from the blinded PE samples increased from 41.8 to 60.5% when the stomacher-centrifugation technique was employed for sample concentration. No (oo)cysts were observed in the method blanks and blind negative PE samples, indicating that, regardless of the processing techniques utilized, laboratory contamination from reagents or materials was effectively controlled.

Recovery of *Cryptosporidium* oocysts and *Giardia* cysts from finished water. Recovery of *Cryptosporidium* oocysts and *Giardia* cysts from 1,000-liter finished-water samples with the Filta-Max system is presented in Table 4. Direct examination of the filter eluate without IMS yielded a mean oocyst recovery of 61.4% and a mean cyst recovery of 65.4% with spike dose concentrations for each organism of 300 to 1,000 (oo)cysts. When these samples where further processed with IMS, oocyst recovery dropped to 44.4% and cysts recovery dropped to 36.3%. When spike dose concentrations of 50 and 100 organisms were used, the entire sample volume was processed with the particle concentrator. Direct examination of subsamples was not performed. (Oo)cysts were then concentrated by IMS,

TABLE 4. Recovery of *Cryptosporidium* oocysts and *Giardia* cysts in 1,000-liter finished-water samples by Filta-Max and method 1623

Approximate (oo)cyst concn (no. of samples)	Mean % (oo)cyst recovery ± SD	
	Direct[a]	Overall[b]
Oocysts		
50 (3)		40.7 ± 21.1
100 (3)		38.6 ± 17.3
300 (2)	58.2 ± 8.5	50.2 ± 5.5
500 (3)	58.8 ± 12.7	50.5 ± 22.9
1,000 (2)	68.6 ± 20.0	43.6 ± 5.7
Cysts		
50 (3)		24.9 ± 4.9
100 (3)		27.8 ± 2.3
300 (2)	69 ± 20.2	41.4 ± 1.3
500 (3)	57.6 ± 10.0	47.3 ± 10.4
1,000 (2)	73.7 ± 2.8	44.3 ± 4.1

[a] Direct refers to direct analysis of the sample concentrate without the IMS step prior to staining.
[b] Overall refers to analysis of the sample including the IMS step prior to staining.

resulting in overall method recovery percentages of 39.7 and 26.4% for *Cryptosporidium* and *Giardia*, respectively.

DISCUSSION

Until the late 1990s, the ICR method in the United States (20) and the SCA method in the United Kingdom (1) were used for *Giardia* and *Cryptosporidium* detection in water. These methods have historically resulted in low and highly variable recovery percentages in part because of the inefficient capture and retention of target organisms on the filter (7, 8, 17). Studies of the robustness of the ICR method have demonstrated that laboratories were unable to recover (oo)cysts seeded into samples at levels in excess of 9,000 organisms (false negatives), while some laboratories reported (oo)cysts in unseeded samples (false positives) (7, 9). Methods for recovery and detection of protozoan parasites have been improved significantly with the introduction of new filter designs for sample collection and the integration of IMS for separating the target organisms from nonspecific interferences (5, 9, 11, 15, 16; McCuin et al., Proc. Am. Water Works Assoc.). The additional requirements for confirmatory DAPI staining and Nomarski-differential interference contrast microscopic examination have also improved the specificity of the method (10, 11). The inclusion of low-level spike doses (~100 [oo]cysts) has also improved the robustness of the methods in determining the effectiveness of laboratory QC measures.

A national survey of 87 U.S. water plants by methods 1622 and 1623 showed an average *Cryptosporidium* recovery across 293 reagent water (ongoing precision and recovery) samples and six laboratories of 42%. The mean *Giardia* ongoing precision and recovery across 186 samples and six laboratories during the ICRSS was 47% (11). The mean *Cryptosporidium* recovery across 430 MS samples from 87 source waters was 43%, while the mean *Giardia* MS recovery across 270 samples from 87 source waters was 53%. In this study, we found that spike doses of ~50 organisms resulted in recovery percentages that met the acceptance criteria for methods 1622 and 1623. The minimum detection values, the levels where seeded (oo)cysts are undetectable by methods 1622 and 1623, have not been determined but could be as low as 10 to 15 (oo)cysts in a 10-liter sample (unpublished data). Precise minimum detection values should be determined in future studies to determine the true lower limits of the methods' assay sensitivity. Method sensitivity information, coupled with (oo)cyst infectivity data, is needed to develop meaningful risk assessment criteria for protozoan pathogens in water supplies (G. DiGiovanni, M. LeChevallier, and M. Abbaszadegan, Proc. Am. Water Works Assoc. Water Quality Technology Conference, abstr. 4F4, 1998).

In 1997, the U.S. EPA proposed a new approach for the development and validation of improved methods known as the PBMS (21). The PBMS approach is an alternative to the use of prescriptive measurement methods that unintentionally create a barrier to the use of innovative environmental monitoring technologies by restricting laboratories to the use of specific products and analytic procedures. The PBMS approach is designed to achieve the following goals: (i) increase method flexibility so that approved methods can be modified without requiring formal U.S. EPA review and approval, (ii) provide a mechanism for independent laboratories to develop and submit new analytic methods for approval, and (iii) expedite the method approval process (21). On a practical level, the implementation of this approach allows individual laboratories to introduce modifications and optimizations of these methods and allow other organizations, including manufacturers of alternative components for the method, to demonstrate the equivalency of modified versions of the method to the U.S. EPA. A primary objective of this study was to compare the Filta-Max system with other sample collection systems used in methods 1622 and 1623 under PBMS testing guidelines. These collaborative trials have established that the Filta-Max system produces data meeting the method acceptance criteria. An alternate Filta-Max elution-and-concentration technique with a stomacher-centrifugation technique was also shown to produce equivalent results for *Giardia* in all of the participating laboratories, but one of four laboratories failed to meet the acceptable mean percentages of *Cryptosporidium* recovery from both the IPR and MS samples. The stomacher-and-centrifugation technique avoids the need to sanitize the Filta-Max wash station and its elution accessories; this modification may be of interest to laboratories processing large numbers of samples and looking for ways to improve productivity. Both methods 1622 and 1623 describe how to conduct PBMS equivalency studies, and laboratories should be encouraged to investigate alternative techniques to improve performance and streamline productivity.

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