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Differences in staining intensities affects reported occurrences and concentrations of *Giardia* spp. in surface drinking water sources

Kerri A. Alderisio^{1,*}, Leah F. Villegas², Michael W. Ware³, Lisa A. McDonald¹, Lihua Xiao⁴, and Eric N. Villegas^{3,*}

¹New York City Department of Environmental Protection, Valhalla and Kingston, NY

²CB&I Federal Services, Inc., Cincinnati, OH 45268

³National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268

⁴National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30329

Abstract

Aim—USEPA Method 1623, or its equivalent, is currently used to monitor for protozoan contamination of surface drinking water sources worldwide. At least three approved staining kits used for detecting *Cryptosporidium* and *Giardia* are commercially available. This study focuses on understanding the differences among staining kits used for Method 1623.

Methods and Results—Merifluor and EasyStain labeling kits were used to monitor *Cryptosporidium* oocyst and *Giardia* cyst densities in New York City's raw surface water sources. In the year following a change to the approved staining kits for use with Method 1623, an anomaly was noted in the occurrence of *Giardia* cysts in New York City's raw surface water. Specifically, Merifluor-stained samples had higher *Giardia* cyst densities as compared with those stained with EasyStain. Side by side comparison revealed significantly lower fluorescence intensities of *Giardia muris* as compared with *G. duodenalis* cysts when labeled with EasyStain.

Conclusions—This study showed very poor fluorescence intensity signals by EasyStain on *G. muris* cysts results in lower cyst counts, while Merifluor, with its broader *Giardia* cyst staining specificity, results in higher cyst counts, when using Methods 1623.

Significance and Impact of Study—These results suggest that detected *Giardia* cyst concentrations are dependent on the staining kits used, which can result in a more or less conservative estimation of occurrences and densities of zoonotic *Giardia* cysts by detecting a broader range of *Giardia* species/Assemblages.

*Corresponding authors' mailing addresses: Eric N. Villegas, Microbial Exposure Branch, Exposure Methods and Measurements Division, National Exposure Research Laboratory. Phone: +1 (513) 569-7017. villegas.eric@epa.gov. Kerri A. Alderisio, Bureau of Water Supply, Division of Water Quality Science & Research, Watershed Impacts and Pathogen Research, New York City Department of Environmental Protection. Phone: +1 (914) 749-5264. kalderisio@dep.nyc.gov.

CONFLICT OF INTEREST

No conflict of interest declared.

Keywords

Drinking water; Protozoa; Environmental/recreational water; Detection

INTRODUCTION

Human infectious types of *Giardia* and *Cryptosporidium* are known to cause diarrheal illness in immunocompetent individuals and can lead to additional stress and illness in the immunocompromised population (Ryan and Caccio 2013; Ryan et al. 2014; Checkley et al. 2015). To minimize risks associated with waterborne disease outbreaks caused by these pathogens, the USEPA promulgated the Long Term 2 Enhanced Surface Water Treatment Rule (LT2) (USEPA 2006) requiring drinking water utilities using surface source waters to monitor for *Cryptosporidium* and *Giardia* using USEPA Method 1623 (USEPA 2005).

Since the promulgation of the LT2 rule, the New York City Department of Environmental Protection (NYCDEP) has routinely monitored, within their 125 square mile watershed, for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts using various approved methods. In late 2001, NYCDEP began monitoring for these organisms using the USEPA approved Method 1623 utilizing Merifluor stain from Meridian Biosciences. Merifluor stain was approved for use with Method 1623 in 1999. In 2000 and 2003, Waterborne, Inc.'s Aqua-Glo and BTF's EasyStain staining kits, respectively, were approved for use in conjunction with Method 1623 (USEPA 2005). Since their approval as two alternative *Cryptosporidium* and *Giardia* staining reagents, EasyStain and Aqua-Glo also became commonly used staining reagents for Method 1623. Because EasyStain has been reported to be specifically designed to reduce or eliminate cross reactivity and background interference (Ferrari 1999), NYCDEP switched to using EasyStain. A side by side performance comparison of the Merifluor and EasyStain was conducted for 15 months at a New York City source water location to determine if there are any potential differences between the two staining procedures.

MATERIAL AND METHODS

Source water monitoring

Two duplicate water samples (50L) were filtered at the Catskill Aqueduct source water outflow location at Kensico Reservoir (CATLEFF) in Westchester County, New York. Water samples were collected and analyzed weekly in accordance with US EPA Method 1623 using the Envirochek high volume (HV) capsule filters (Pall, Corp., Cortland, NY, USA) (USEPA 2005) for 66 consecutive weeks. Each of the samples was processed and stained with either Merifluor (Meridian Biosciences, Cincinnati, OH, USA) or EasyStain (BTF Precise Microbiology, Pittsburgh, PA, USA) for comparison as described below. The initial demonstration of capability testing for EasyStain was performed with a mean recovery 54.25% $\pm$ 4.11(SD) for *Giardia* cysts and 66.5% $\pm$ 2.64 (SD) for *Cryptosporidium* oocysts.

Giardia cysts

Two assemblages of *G. duodenalis* were used in this study: WB (Assemblage A) and H3 (Assemblage B). WB trophozoites were obtained from American Type Culture Collection (ATCC 30957; Manassas, VA, USA). The H3 isolate was originally obtained from Waterborne, Inc. (New Orleans, LA, USA). *G. muris* cysts were originally obtained from Case Western University (Hayes et al. 2003) and propagated at the USEPA animal facilities. Experimentally exposed CF1 mice or Mongolian gerbils (Charles River Laboratories, Wilmington, MA, USA) were used for *G. muris* (Roberts-Thomson et al. 1976) or *G. duodenalis* (Ware et al. 2013) cyst production. Cysts collected from infected animals were purified by sieving, sucrose flotation, and using a continuous Percoll gradient as previously described (Sauch 1984). Because of the limited number of *G. duodenalis* WB cysts recovered, they were not Percoll purified. All animal studies were approved and overseen by the Cincinnati USEPA Institutional Animal Care and Use Committee. Cysts used in this study were within two weeks of collection.

Cryptosporidium oocysts

Cryptosporidium parvum oocysts (Iowa II strain) were purchased from Wisconsin State Laboratory of Hygiene (Madison, WI, USA). Flow sorted oocysts were performed to obtain spike samples as described in USEPA Method 1623 (USEPA 2005). Oocysts were labeled as described in the staining procedures below, prior to flow cytometric and microscopic analyses.

Giardia staining procedures

Each tube containing 3×10^4 *G. duodenalis* cysts Assemblage A, B, or *G. muris* species were labeled with Aqua-Glo (Waterborne, Inc.), Merifluor (Meridian Biosciences, Cincinnati, OH, USA), or EasyStain (BTF Pty. Ltd., North Ryde, NSW, Australia) per manufacturer's instructions. Briefly, cysts were labeled for 45 min at room temperature in the dark with 150 μ l of either EasyStain or Merifluor reagent at the concentration provided, except for Aqua-Glo (20X), which was diluted in reagent water to 1X before adding the same amount to the sample. After staining, the samples were then centrifuged and the supernatant was aspirated. EasyStain samples were fixed for 2 min by adding 300 μ l ice cold fixing buffer, and once again centrifuged and aspirated. All samples were counterstained for 1 min with 4',6-diamidino-2-phenylindole (DAPI; Sigma) at $400 \text{ ng} \cdot \text{ml}^{-1}$, except for the EasyStain samples which was at $2 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ DAPI. Finally, tubes were centrifuged and aspirated as described above and then suspended in 300 μ l reagent water. Unused labeled cysts were kept on ice in the dark, for no longer than 30 min, until analysis by flow cytometry.

Flow cytometry

Fluorescence intensities of the conjugated antibody staining reagents were measured using a FACS AriaII (Becton Dickinson, San Jose, CA, USA) equipped with a UV laser using PBS as a sheath fluid. Briefly, the forward and side scatter parameters were adjusted to separate cysts above background noise. A gate was drawn around the cysts, which were further analyzed for FITC and DAPI fluorescence intensities as previously described (Ware and Schaefer 2005). Because *G. duodenalis* (Assemblage A) cysts were not Percoll purified and

had a heavy bacterial load, a FITC threshold was added to filter out interfering debris allowing for the detection of stained cysts only. The mean fluorescence intensity of at least 4000 events within the drawn gate were then reported for the FITC channel.

Data and statistical analyses

Data analyses were performed using Excel 2016 (Microsoft, Redmond, WA, USA). Statistical analyses were performed using SigmaPlot (Version 13, Systat Software, Inc., San Jose, CA, USA). Student's *t*-test or analysis of variance (ANOVA) was performed to determine significance among the different conditions described in this study. A Shapiro-Wilk Normality test was conducted to determine if the data sets were normally distributed. Statistical power conducted with the reported data set is at least 0.095. A *p* value of less than 0.05 was considered significant.

RESULTS

Occurrence and concentration of *Giardia* and *Cryptosporidium* in source water

A total of 66 side-by-side samples were analyzed using Merifluor and EasyStain over the course of 66 weeks. *Cryptosporidium* occurrence and oocyst concentrations were assessed using either Merifluor or EasyStain in the duplicate samples (Table 1). Mean concentration of *Cryptosporidium* oocysts and number of positive samples labeled with Merifluor (0.12 oocysts • 50 l⁻¹ and 8, respectively) were slightly lower when compared to the corresponding EasyStain samples (0.20 oocysts • 50 l⁻¹ and 12, respectively). In total, Merifluor labeled slides yielded a total of 8 single oocysts, while the EasyStain slides resulted in 13 oocysts (11 single oocyst samples and one sample with 2 oocysts) from each of the untreated positive water samples. While the data suggest that EasyStain yielded more positive *Cryptosporidium* samples and slightly higher mean oocyst concentration, the differences between the results were not statistically significant for *Cryptosporidium*. Therefore, the remainder of the study focused solely on *Giardia* cyst concentrations and occurrences as described below.

By contrast, *Giardia* results revealed a marked difference in concentration and occurrences of cysts using EasyStain, whereby most samples resulted in lower concentrations and total numbers of *Giardia* cyst slides that were positive when using EasyStain as compared with samples processed using Merifluor (Table 1 and Figure 1). Mean concentrations of *Giardia* cysts and the number of positive samples with Merifluor stain (2.85 cysts • 50 l⁻¹ and 54, respectively) were higher when compared with the corresponding samples stained with EasyStain (1.06 cysts • 50 l⁻¹ and 29; *p*<0.001 and *p*<0.0001, respectively) (Table 1). During the time period studied, slides labeled with Merifluor yielded a total of 188 cysts, while the EasyStain slides resulted in 70 cysts from the 66 samples examined. Additionally, cyst concentrations from the two stains did not correlate well (*r*²= 0.391) with a higher overall count skewed toward the samples labeled with Merifluor (Figure 2A). This lack of strong correlation differs from duplicate samples processed with the same sample, as observed when matrix spikes and matrix spike duplicate recoveries were analyzed. Results from both duplicate samples were very similar, with a correlation coefficient of 0.092 (*r*²= 0.853) (Figure 2B), indicating that the differences in concentrations observed between Merifluor

and EasyStain were not due to Method variation. Overall, the *Giardia* cyst concentrations and occurrence differed in surface water samples depending on the labeling kit used.

Seasonal variation of *Giardia* cysts in NYCDEP source water

During the study, 66 weeks of sampling occurred at the Catskill source water which allowed for a determination of potential seasonal effects on occurrences and concentrations of *Cryptosporidium* oocysts and/or *Giardia* cysts in this watershed. Seventeen additional duplicate samples analyzed with both stains were collected at three other source water locations and were included in the seasonal analysis for a total of 83 paired samples. As shown in Table 2, seasonal variations in cysts were observed using either staining kit (Table 2). Both labeling reagents produced the highest mean concentrations of *Giardia* cysts in the winter (Merifluor = 4.55 cysts • 50 l⁻¹ and EasyStain = 1.80 cysts • 50 l⁻¹), and the lowest in the summer (Merifluor = 1.46 cysts • 50 l⁻¹ and EasyStain = 0.08 cysts • 50 l⁻¹). Moreover, the difference in counts between the two labeling reagents was greater during the winter when *Giardia* cyst total numbers were higher compared to the other three seasons. The mean difference between the cyst counts for Merifluor and EasyStain in the winter was 2.75 cysts, whereas for Spring, Summer and Fall, the count differences were 1.40, 1.38 and 1.08 cysts respectively. Together, regardless of the staining kit used, there were significant differences in the cyst concentrations detected from different seasons ($p < 0.001$, ANOVA), with the winter season yielding the highest *Giardia* concentrations detected.

Flow cytometric analysis of *Giardia* spp. cysts

To determine the staining performance of the various approved staining kits used for USEPA Method 1623, *G. duodenalis* Assemblage A and B as well as *G. muris* cysts were stained and analyzed by flow cytometry. As shown in Figure 3, *G. duodenalis* (Assemblage A and B) (Figures 3 A – F, J and K) stained intensely, approaching 200,000 mean fluorescence intensity units (MFI). The staining profiles of both Assemblages were similar to each other when labeled with either labeling kit, however, *G. duodenalis* cysts stained with EasyStain had the highest staining intensities with an MFI equal to or greater than 200,000 (Figure 3 J and K). The observed fluorescence intensity of *G. duodenalis* cysts stained by Aqua-Glo had the lowest MFI as compared with EasyStain and Merifluor (123,000 vs >178,000) (Figure 3 J – L). *G. duodenalis* labeled cysts were also easily detected by epifluorescent microscopy regardless of the labeling reagents used. By contrast, the MFI for *G. muris* cysts was an order of magnitude lower than what was observed with *G. duodenalis* cysts (< 24,000 MFI) regardless of the staining kit used (Figure 3 G – I, and L). Furthermore, *G. muris* cysts stained with EasyStain were very difficult to detect by microscopy. This sample also had the lowest observable fluorescence (lowest MFI) with levels that were only slightly higher than unstained cysts (1,027 and 373, respectively), among all three staining kits tested. Despite the low fluorescence intensities of cysts stained with Merifluor and Aqua-Glo, these cysts were still detectable by fluorescence microscopy. The observed DAPI staining results, by microscopy and MFI by FACS, were not consistent even within the same cyst lot.

DISCUSSION

The importance of obtaining accurate pathogen concentration in drinking water sources is crucial to estimating human health risks associated with potential drinking water related disease outbreaks. For example, as described in the LT2 rule, *Cryptosporidium* oocyst concentration using USEPA Method 1623 is critical in determining Bin classification for various drinking water utilities. Any changes in the method could have a significant impact in the overall water quality assessment of the source water used for drinking water. In this study, we did not see any statistically significant difference in *Cryptosporidium* oocyst concentration regardless of the staining kit used to enumerate the oocysts (Table 1). By contrast, a significant difference in *Giardia* cyst concentration was noted in the watershed samples studied when Merifluor and EasyStain staining kits were compared. The use of Merifluor resulted in significantly more positive samples and higher concentrations of cysts than EasyStain (Table 2 and Figure 1). Also, comparisons of the side by side *Giardia* sample recovery demonstrated that the samples stained with different kits were not comparable, whereas duplicate samples processed with the same staining kit were similar (Figure 2).

When three USEPA approved staining kits were used to directly label *G. duodenalis* assemblages A and B, and *G. muris*, all staining kits performed well with *G. duodenalis* assemblages A and B. Unexpectedly, we observed an overall lower fluorescence intensity with *G. muris* cysts. In fact, unlike Merifluor and Aqua-Glo, EasyStain did not result in a sufficient fluorescence intensity to easily detect *G. muris* by fluorescence microscopy or FACS (Figure 3), which may account for the overall reduction in the reported *Giardia* cyst concentrations observed (Table 1).

A seasonal variation of *Giardia* cyst concentration was also observed in this study with winter and spring seasons having the highest levels of cysts during the study, regardless of the staining kits used. These results were not surprising since previous studies eluded to a potential seasonal variation in parts of North America with the highest cyst concentration reported to occur in the fall and winter season in the US (USEPA 1999). Studies by Siqueira-Castro (2016) recently reported that ciliated free-living protozoa, *Euplotes aediculatus* and *Sterkiella cavicola*, which are found in fresh water and wastewater treatment plants (e.g., activated sludge), are capable of ingesting *Giardia* cysts. This observation suggests that predation of *Giardia* cysts by ciliated protozoa may play a role as a natural biological removal process of cysts in surface and waste waters. Moreover, since free living ciliated protozoa tend to be more abundant in warmer temperatures (Hadas and Berman, 1988), this increase in abundance of ciliated protozoa could also account for the seasonal trends we reported in this study, with lower cysts and oocysts concentrations observed during the warmer months. Given the zoonotic nature of *Giardia duodenalis*, it is difficult to predict the source(s) of *Giardia* contamination in this watershed without additional analyses, such as molecular genotyping. Therefore, attempts were also made to determine species and Assemblages of *Giardia* cysts detected by PCR sequence typing. Unfortunately, most *Giardia* cysts detected on the slides were amorphous or empty and thus nucleic acid materials may not have been readily available for subsequent genotyping (data not shown). Nevertheless, slides that were positive for *Giardia* cysts were further processed using the off-slide genotyping procedures as previously described (Ware et al. 2013). Two sets of

paired samples (4 slides) positive for *Giardia* cysts by both Merifluor and EasyStain were analyzed by PCR, however, no PCR amplicons were detected even after 10 replicate analyses.

Giardia duodenalis Assemblages A and B are known zoonotic pathogens and therefore infectious to humans (Feng and Xiao 2011). All approved staining kits for USEPA Method 1623 are able to detect these Assemblages, as shown in this study. However, IMS recovery and staining of non-human species, Assemblages and genotypes are largely unknown. The Merifluor antibody, consisting of IgG and IgM isotypes, can potentially behave as a pan-specific antibody capable of reacting to a broad range of *Giardia* spp. and Assemblages (Rodgers et al. 1995; Kostopoulou et al. 2015). As shown in this study, Merifluor can indeed detect at least three different Assemblages/species *G. duodenalis* Assemblages A/B, and *G. muris*. This characteristic can also result in increased reactivity with environmental contaminants typically found in surface waters, which can result in staining interference and/or false positive detections (Rodgers et al. 1995). By contrast, EasyStain, which is an IgG isotype, is relatively more specific and potentially has lower possibility for cross reactivity (Ferrari 1999) and thus cyst concentration estimates derived from using the EasyStain kit estimates will be more specific to species known to cause disease in humans. To the best of our knowledge, this is the first study demonstrating the differences between Merifluor and EasyStain specificity when used for assessing *Giardia* cyst concentrations in surface water. Additional studies however, are needed to completely understand the reactivity of these kits to various *Giardia* species and Assemblages.

In general, when compared to occurrence data gathered using EasyStain, utilities using Merifluor may appear to have higher *Giardia* cyst densities in their respective watersheds. Consequently, stain type should be considered when comparing nationwide data among multiple laboratories. Utilities should also be aware of the potential data shift that may occur after changing stains. In this study, the performance-based criteria for Method 1623 were met; however, the database associated with *Giardia* density levels throughout the US may change significantly depending on the staining kits employed. The difference in the counts between the two stains may be inconsequential if EasyStain is specifically designed and successful in targeting only *Giardia* species most likely to cause human infection. The types of *Giardia* that do not cause infection in humans are less likely to be detected. Conversely, emerging zoonotic *Giardia* species, Assemblages, or genotypes could also be overlooked. Although Merifluor may overestimate the actual risk of *Giardia* to public health by detecting a broader range of *Giardia* species, including non-pathogenic species and Assemblages, it could still be used to assess efficacies of treatment processes used, and more importantly, as a more conservative approach at determining overall *Giardia* cyst densities in a watershed.

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Figure 1. Concentrations of *Giardia* spp. cysts at the Catskill Aqueduct source water. Duplicate 50 l samples were collected for 66 consecutive weeks and analyzed using USEPA Method 1623. Either A) Merifluor or B) EasyStain kits were used to measure cyst concentration.

Figure 2.

Comparison of A) *Giardia* cyst concentration using Merifluor and EasyStain staining kits or B) overall present recoveries collected for the matrix spike (MS) and matrix spike duplicate (MD). A weak correlation ($r^2 = 0.391$) was observed with cyst concentrations from the two stains used in this study with a higher overall cyst counts in samples labeled with Merifluor. A strong and statistically significant correlation ($r^2 = 0.853$; $p < 0.0001$) was observed between MS and MD.

Figure 3.

Flow cytometric analysis of the *Giardia duodenalis* Assemblage A and B and *G. muris* cysts. *Giardia* cysts were stained with either EasyStain, MeriFluor, or AquaGlo. Histogram analyses of the mean fluorescence intensity of *G. duodenalis* Assemblage A (panels A – C), Assemblage B (panels D – F), and *G. muris* (panels G – I) were stained with EasyStain (panels A, D, and G), MeriFluor (panels B, E, and H), or Aqua-Glo (panels C, F, and I) are shown. Unfilled histogram plots represent unstained *Giardia* cysts while solid filled histogram plots represent cysts stained with the various staining kits. Histogram markers depicts samples considered to be positively stained with the various staining kits. Panel J, K, and L shows mean fluorescence intensities *G. duodenalis* Assemblage A, Assemblage B, and *G. muris* stained with the various staining kits, respectively.

Table 1*Giardia* cyst and *Cryptosporidium* oocyst detected in surface waters

	Merifluor	EasyStain
<i>Giardia</i> spp.		
Mean concentration (cyst • 50 l ⁻¹)	2.85 *	1.06 *
No. positive	54/66 (82%) **	29/66 (44%) **
<i>Cryptosporidium</i> spp.		
Mean concentration (oo cyst • 50 l ⁻¹)	0.12	0.20
No. positive	8/66 (12%)	12/66 (18%)

* , p<0.001;

** , p<0.0001

Table 2Seasonal variations of *Giardia* spp. cysts concentrations^{*}

Season	No. Sample	Merifluor	EasyStain
Winter	12	4.55	1.80
Spring	29	2.45	1.05
Summer	13	1.46	0.08
Fall	29	1.58	0.50

^{*} Concentrations are number of cysts 50 l^{-1} . $P < 0.001$.