Improved cell culture immunofluorescent assay for detection of infectious Cryptosporidium spp. in prefinished water

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The New York City Department of Environmental Protection's (NYC) Hillview Reservoir is an uncovered prefinished water storage reservoir post-ultraviolet (post-UV) light disinfection. As an additional tool to the detection of total (viable and nonviable) oocysts using Methods 1623/1623.1, the performance of an improved cell culture immunofluorescent assay (CC-IFA) for the specific detection of infectious oocysts in this NYC reservoir matrix was evaluated. Mean CC-IFA matrix spike recovery of infectious Cryptosporidium parvum and Cryptosporidium hominis oocysts was comparable to the mean Method 1623 recovery of total oocysts, ranging from 29 to 46%. Experiments using ≤5 C. parvum or ≤10 C. hominis viable oocyst spikes prepared using flow cytometry indicated that the reservoir matrix did not adversely affect CC-IFA detection. Therefore, the improved CC-IFA appears useful for the detection of environmentally relevant, low numbers of infectious oocysts in the reservoir matrix and for the refined assessment of Cryptosporidium risk.

KEYWORDS
CC-IFA, cell culture, Cryptosporidium, immunofluorescent assay, infectivity, risk

1 | IMPORTANCE

The detection of infectious Cryptosporidium in water is critical for accurately assessing public health risk and for identifying appropriate management actions needed to protect water supplies. Cryptosporidium parvum and Cryptosporidium hominis are responsible for the majority of human cryptosporidiosis, and it is important that both species be evaluated. An improved cell culture immunofluorescent assay (CC-IFA) method able to sensitively detect infectious oocysts of these strains will inform risk models and guide best management practices. The CC-IFA method evaluated in this study demonstrated acceptable recovery in the matrix tested and was successful at detecting environmentally relevant numbers of oocysts. This study represents one of the most comprehensive evaluations of the CC-IFA method for the detection of low numbers of infectious C. parvum and C. hominis in environmental water samples.

2 | INTRODUCTION

Cryptosporidium spp. oocysts can cause the illness cryptosporidiosis in a broad range of animal hosts. In humans, cryptosporidiosis is characterized by infection of the digestive tract's epithelial cells, resulting in acute diarrhea that may be either self-limiting in the immunocompetent or cause serious complications for immunocompromised individuals. Methods for the detection and enumeration of Cryptosporidium spp. oocysts in water samples have improved greatly...
over the decades, culminating in the current versions of the U.S. Environmental Protection Agency (USEPA) Methods 1623/1623.1 (USEPA, 2005, 2012). While much improved in the recovery of oocysts, these methods do not identify the species of Cryptosporidium detected or determine oocyst viability or infectivity and may therefore overestimate the risk to public health. Not all Cryptosporidium species infect humans (Nichols, Connelly, Sullivan, & Smith, 2010; Ruecker et al., 2012), and not all oocysts present in water are viable and infectious (LeChevallier et al., 2003; Lalancette et al., 2012).

The New York City Department of Environmental Protection (NYC) Bureau of Water Supply has an uncovered prefinished water storage reservoir postultraviolet (post-UV) light disinfection. Previous research has demonstrated that oocysts that have passed through UV treatment, and are hence no longer infectious, are still detected by Method 1623 (Bukhari, Hargy, Bolton, Dussert, & Clancy, 1999; Clancy, 2000). Therefore, in addition to detecting oocysts using Method 1623, the performance of an improved CC-IFA for detect infectious oocysts present in the NYC reservoir matrix was evaluated. The NYC Catskill/Delaware UV disinfection process is designed for up to 3-log inactivation of Cryptosporidium and is a very effective treatment for rendering oocysts noninfectious. NYC has an active wildlife management program for the reservoir, which includes but is not limited to water bird surveys, dispersion techniques, live trapping, bird deterrent wires, and daily sanitary surveys. However, if any oocysts are introduced directly into the reservoir by wildlife (birds/small mammals) post-UV, they could potentially be infectious. Cryptosporidium CC-IFA can determine oocyst infectivity, and genotyping of cell culture infectious foci can determine the Cryptosporidium species, providing more useful information than Method 1623 alone.

In this study, samples were analyzed for both C. parvum and C. hominis recovery using USEPA Methods 1623 and CC-IFA from spiked 100-L reservoir water matrix samples at two laboratories. Experiments were also performed using 100-L reservoir water samples spiked with low numbers of oocysts prepared using flow cytometry to evaluate the ability of the CC-IFA method to detect environmentally relevant numbers of infectious oocysts. Based on the results of this study, CC-IFA appears suitable for this reservoir matrix and for the refined assessment of Cryptosporidium risk.

3 | MATERIALS AND METHODS

3.1 | Sources and quality control of Cryptosporidium oocysts

Mouse-propagated C. parvum oocysts (Iowa isolate) were obtained from Waterborne Inc. (New Orleans, Louisiana). C. hominis TU728 isolate oocysts propagated in gnotobiotic pigs were obtained from Tufts University (North Grafton, Massachusetts). Oocysts used for infectivity assays were less than 6 weeks old (postshedding), and 70% of lots were 4 weeks old or less. Oocysts from the same lot were shipped to two analytical laboratories at the same time for each round of testing, as noted below.

3.2 | Enumerating oocysts

Oocysts were enumerated and sorted by flow cytometry at the Wisconsin State Laboratory of Hygiene (Madison, Wisconsin). Oocysts were sorted into individual microcentrifuge tubes for each round of experiments. The average relative standard deviation for flow cytometry-enumerated oocyst doses of 1–100 was 1.2% (n = 180).

3.3 | Control infections

Five replicate trip control tubes of oocysts at each spike level were included in each trial. Trip controls were stored at 4°C at the NYC laboratory until matrix spike (MS) samples were collected and partially processed. Both MS and trip control samples were shipped to the University of Texas Health Science Center at Houston School of Public Health in El Paso (UTHealth) laboratory and analyzed using CC-IFA alongside infection controls to determine whether the shipping process affected oocyst infectivity.

The infectivity of each lot of oocysts was determined before each set of associated trials. Direct inoculation of human ileocecal adenocarcinoma (HCT-8) cell monolayers was performed and the enumeration of infectious foci was determined with IFA and fluorescence microscopy. On the basis of preliminary trials with the CC-IFA conditions used in this study, oocyst lots were only used for experiments if their mean infectivity in five replicate monolayers was >40% for C. parvum and >20% for C. hominis.

3.4 | Cell culture maintenance

Cell culture procedures were a modification of those used by Johnson, Di Giovanni, & Rochelle, 2012, which is identical to Standard Method 9711 D (Rochelle, Di Giovanni, et al., 2012), incorporating some procedures of King, Keegan, Phillips, Fanok, & Monis, 2012. Detailed procedures were as follows. Monolayers of the HCT-8 cell line (ATCC CCL-244; American Type Culture Collection, Rockville, Maryland) were maintained in 150 cm² flasks and passaged twice a week in cell culture maintenance medium. The maintenance medium consisted of RPMI-1640 medium with GlutaMAX (Invitrogen, Carlsbad, California) containing 5% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Cells were passaged by rinsing the monolayer with 10 mL of RPMI-1640 basal medium and then adding 10 mL of
0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Thermo Fisher Scientific, Waltham, Massachusetts) and incubating for 10 min at 36°C to release the cell monolayer from the flask. Trypsin was inactivated and removed by adding an equal volume of cell culture maintenance medium, centrifuging the cells at 160 × g for 5 min and resuspending the cells in the cell culture maintenance medium. Cells were enumerated using a hemacytometer. Each 150 cm² flask containing 50 mL fresh maintenance medium was inoculated with 6.75 × 10⁶, 5.25 × 10⁶ or 3.75 × 10⁶ cells for 2-, 3-, or 4-day passage, respectively. The cells were maintained in a humidified incubator at 36°C and 5% CO₂. Cells were not used beyond passage 30. Separate biological safety cabinets and incubators were used for uninfected stock cells and infected monolayers.

To prepare monolayers for infection, a stock flask of HCT-8 cells was split into two 150-cm² flasks of fresh maintenance medium. One flask (the assay flask seeded with 6.75 × 10⁶ cells) was used to set up 48-well plates (Corning Inc., Corning, New York) for the CC-IFA detection assay, while the second flask was used for subsequent cell passages. The assay flask was incubated for 42–52 h to achieve 80–100% confluence, and the monolayer was then removed from the flask by trypsinization as described above, with resuspension of cells in the monolayer seeding medium. Monolayer seeding medium was the same as the maintenance medium except that FBS was increased to 10% and containing kanamycin (100 μg/mL); 48-well plates were inoculated with 1 mL of monolayer seeding medium containing 2.5 × 10⁵ cells/mL. After 42–52 h incubation at 36°C, confluent monolayers were inoculated with oocysts.

### 3.5 | Matrix sample field collection

Samples for the 100 oocyst spikes were collected in accordance with USEPA Method 1623. In summary, before sample capsule attachment, the apparatus was connected to a wall-mounted manifold and flushed with 80 L of matrix water. The manifold delivered matrix water from a submersible pump in the forebay of the Hillview Reservoir outflow to four sample taps. This allowed for multiple apparatuses to be used concurrently. An Envirochek HV (high volume) sampling capsule (Pall Corp., Port Washington, New York) was installed inline between the flow meter and the pressure gauge. Pressure remained well below the 60 psi threshold throughout sample filtration. Two identical sampling apparatuses were simultaneously flushed and then deployed to expedite collection times. The 10-L bulk water samples were collected into low-density polyethylene plastic cubitainers from an extra sample tap on the manifold. Four 90-L bulk water samples were collected into this manner for each round of sampling. For each low oocyst dose MS sample, 100 L of field water was filtered, and two apparatuses were deployed simultaneously to increase collection efficiency of the 15 filters required for each round. All samples were transported in coolers on wet ice back to the field office for shipping to the NYC laboratory in Kings- ton, New York. For each trial, 100-L ongoing precision and recovery (OPR) and MS samples were processed using Method 1623. A volume of 90 L of water (laboratory reagent grade or matrix) was filtered through an Envirochek HV filter (Pall Corp.) in the lab (OPR) and in the field (MS), and the final 10-L portion was spiked in the laboratory with the appropriate dose of flow-sorted oocysts. Samples were stained the following day using either Merifluor (Meridian Bioscience, Cincinnati, Ohio) or EasyStain (BTF, North Ryde, New South Wales).

### 3.6 | Oocyst pretreatment

For matrix-free samples, individual microcentrifuge tubes containing 150 μL of molecular grade water and the desired number of flow-sorted oocysts were pretreated before inoculating HCT-8 monolayers. To each tube of flow-sorted oocysts, 150 μL of freshly prepared acidified Hanks' balanced salt solution (AHBSS) adjusted to pH 2.0 and containing 2% trypsin (type II-S porcine pancreas, catalog no. T7409; Sigma Chemical Co., St. Louis, Missouri) (AHBSS/trypsin; final concentration 1% trypsin), was added, and samples were incubated for 1 h at 37°C with vortex mixing every 15 min (Di Giovanni & LeChevallier, 2005).

For low-oocyst-dose MS experiments, unseeded 100-L field water samples were processed by the Department of Environmental Protection laboratory using Method 1623. To minimize loss of oocysts due to sample transfer, supernatants were transferred into tubes containing 150 μL AHBSS/2% trypsin. Tubes were vortexed for 10 s and again separated by magnetic particle concentration per Method 1623. To expedite collection times (Di Giovanni & LeChevallier, 2005). For low-oocyst-dose MS samples, individual microcentrifuge tubes containing 150 μL of molecular grade water and the desired number of flow-sorted oocysts were pretreated before inoculating HCT-8 monolayers. To each tube of flow-sorted oocysts, 150 μL of freshly prepared acidified Hanks' balanced salt solution (AHBSS) adjusted to pH 2.0 and containing 2% trypsin (type II-S porcine pancreas, catalog no. T7409; Sigma Chemical Co., St. Louis, Missouri) (AHBSS/trypsin; final concentration 1% trypsin), was added, and samples were incubated for 1 h at 37°C with vortex mixing every 15 min (Di Giovanni & LeChevallier, 2005).

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with magnetic beads; samples were vortexed for 10 s, beads separated as above, and the sample supernatants pooled.

After AHBSS/trypsin treatment, oocysts were washed twice by adding cell culture inoculation medium, centrifuging at 13,000 × g for 2 min, and then discarding the supernatant as previously described (Sifuentes & Di Giovanni, 2007). Inoculation medium consisted of RPMI-1640 medium with GlutaMAX containing 1% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), 20 mM HEPES buffer, kanamycin (100 μg/mL), 50 mM glucose, 8.8 μg/mL ascorbic acid, 0.25 μg/mL folic acid, 1.0 μg/mL 4-aminobenzoic acid, 0.5 μg/mL calcium pantothenate, and 200 μg/mL freshly prepared bovine bile (catalog no. B-3883; Sigma Chemical Co.). The final pellet of oocysts was resuspended in 500 μL cell culture inoculation medium and used to inoculate monolayers.

3.7 Cell culture immunofluorescent assay

Cell culture seeding medium was removed from HCT-8 monolayers grown to at least confluence in 48-well plates, and 500 μL of inoculation medium was added to each well to prevent the monolayers from drying out during the inoculation procedure. Following oocyst pretreatment as described above, samples were inoculated onto monolayers, resulting in a final volume of 1 mL/well. Oocysts were centrifuged onto monolayers at 400 × g using a swinging bucket rotor with microplate carriers for 5 min without brake (King et al., 2012). The inoculated 48-well plates were then incubated at 36°C for 64–72 h in a 5% CO₂ humidified incubator.

After incubation, inoculation medium was removed from the wells, and the monolayers were immediately fixed with 250 μL methanol for 10 min. Methanol was removed, and monolayers were allowed to air dry. Monolayers were incubated in 250 μL blocking buffer (2% goat serum and 0.002% Tween-20 in PBS) for 30 min on a rocking platform at room temperature. After removal of the blocking buffer, 100 μL of 8 μg/mL unlabeled rat anti-Cryptosporidium sporozoite antibody (SporoGlo, catalog no. A600UN, Waterborne Inc., New Orleans, Louisiana), prepared in PBS, was added to monolayers. Plates were incubated in a humidified container and incubated on a rocking platform at room temperature for 60 min. After two washes with 250 μL of PBS, 100 μL of goat anti-rat IgG Fluorescein isothiocyanate (FITC)-labeled antibody (catalog no. F6258, Sigma Chemical Co.) diluted 1:300 in PBS was added to monolayers. Plates were placed in a humidified container, protected from light, and incubated on a rocking platform at room temperature for 60 min. The antibody was removed with two washes of 250 μL PBS and 150 μL Method 1623/1623.1 mounting medium added. Plates were examined using epifluorescence microscopy (485 nm excitation, 520 nm emission) with an inverted epifluorescence microscope.

Infection detected by IFA was defined as a monolayer that contained at least one focus of life stages. A focus of life stages was defined as at least three life stages within an area ≤175 μm in diameter. An individual life stage was defined as an intracellular life cycle stage ≥1 and ≤10 μm in

![Flow chart for 100 oocyst and low dose spiked samples analyzed by Method 1623 and CC-IFA. CC-IFA: cell culture immunofluorescent assay; HTC-8: human ileocecal adenocarcinoma; MS: matrix spike; OPR: ongoing precision and recovery; PBS: phosphate buffered saline](image-url)
results ranged from 37 to 60% infective oocysts, while the oocysts using Method 1623 (46 ± 15%) was higher than the OPR recovery of infectious oocysts at 44 ± 16%, while the MS mean recovery of total oocysts using Method 1623 was lower at 29 ± 17%. The CC-IFA MS recoveries of infectious oocysts ranged from 21 to 68%, while Method 1623 MS recoveries of total oocysts ranged from 9 to 49%.

Comparison of recoveries showed statistically significant higher recoveries for C. parvum than C. hominis for Method 1623 and CC-IFA OPR samples (p < 0.05) but not for MS samples.

4.5 | Low dose spiking

Experimentation with low doses of oocysts is critical since most raw drinking water samples that test positive for Cryptosporidium have very low numbers of oocysts present (Ongerth, 2013). Low dose spike levels were 1, 3, and 5 oocysts for C. parvum and 3, 5, and 10 oocysts for C. hominis. Ten replicate MS samples, matrix-free infection controls, and trip controls were analyzed for each dose during each trial. The CC-IFA MS detection of infectious C. parvum oocysts was comparable to matrix-free control samples at each of the low doses tested (Table 3). Of the 30 samples tested with 5 or fewer C. parvum oocysts, the MS samples had equal positivity compared with the infectivity control samples, each having 25 (83%) positive wells. Moreover, at the lowest dose of 1 oocyst, infection in MS samples was as successful, or even more sensitive, than the control samples, with 7 of 10 wells positive.

The detection of infectious C. hominis oocysts in MS samples was also similar to control samples at each of the low doses evaluated (Table 3). Overall, the 30 low dose C. hominis MS samples with 10 or fewer oocysts resulted in the same positivity as C. parvum MS testing (25 wells, also 83%). When compared with control samples, C. hominis MS was slightly lower than infection control samples (28 wells) and slightly higher than trip controls (24 wells).
4.6 | Genotyping CC-IFA foci of infection

Any samples positive for infectious *Cryptosporidium* using the CC-IFA method would ideally be tested further to determine whether the infection resulted from a species or genotype pathogenic to humans. Therefore, to evaluate this approach, individual foci were picked from control and MS monolayers and subjected to genotyping. One focus was sampled from each of three infection control monolayers of *C. parvum* and *C. hominis* TU502 (six total control foci). In addition, two foci from each of the nine MS monolayers from *C. hominis* samples were collected for genotyping (18 total MS foci). Three monolayer areas that appeared to be negative for foci were also tested.

All foci picked from the *C. parvum* and *C. hominis* infection controls and the *C. hominis* MS samples (24 total) were amplified at the *hsp70* target. Sequence analysis of PCR amplicons from the *C. parvum* and *C. hominis* foci matched their appropriate control sequences. While every attempt was made to avoid areas of foci, two of the three samples collected in what were believed to be clear zones of the monolayer were also amplified. Because of the high number of foci present on the monolayers, it was very difficult to avoid foci. In field application, especially with a finished water sample with only one or two foci present per monolayer, an uninfected zone of the monolayer for use as a negative control would not be difficult to locate. However, this does identify an issue for further investigation when dealing with higher densities of foci. More importantly, it is clear that the focus genotyping approach is sensitive and not adversely affected by the Hillview Reservoir matrix.

### TABLE 1  *Cryptosporidium parvum* percent infectivity using CC-IFA and percent recovery using Method 1623—100 oocyst, 90 L + 10 L spikes—rounds 1 and 2

| Round event* | CC-IFA stock infectivity (mean %) | CC-IFA OPR recoverya (mean %) | Method 1623 OPR recovery (mean %) | CC-IFA MS recovery infectious oocysts b (mean %) | Method 1623 MS recovery total oocysts (mean %) |
|---------------|-----------------------------------|-------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------------------|
| Round 1       |                                   |                               |                                   |                                               |                                             |
| 1             | 62 89 68                           | 60 60                         |                                   |                                               |                                             |
| 2             | 79 84 66                           | 57 57                         |                                   |                                               |                                             |
| 3             | 70 644 66                         | 57 57                         |                                   |                                               |                                             |
| Round 2       |                                   |                               |                                   |                                               |                                             |
| 4             | 69 100 64                         | 39 39                         |                                   |                                               |                                             |
| 5             | 74 87 74                           | 42 43                         |                                   |                                               |                                             |
| 6             | 80 108 79                         | 37 28                         |                                   |                                               |                                             |
| Total mean all six events ± SD (%) | 72 ± 12 | 89 ± 15 | 70 ± 6 | 46 ± 15 | 46 ± 12 |

*Note.* CC-IFA: cell culture immunofluorescent assay; MS: matrix spike; OPR: ongoing precision and recovery; SD: standard deviation.  
a Events 1–3 oocyst lot 1; events 4–6 oocyst lot 2.  
b Sample size per each of six events.  
c CC-IFA % recovery calculated using stock control as baseline infectivity.  
d Approximately 50 μL leaked during shipment.

### TABLE 2  *Cryptosporidium hominis* percent infectivity using CC-IFA and percent recovery using Method 1623—100 oocyst, 90 L + 10 L spikes—rounds 1 and 2

| Round event* | CC-IFA stock infectivity (mean %) | CC-IFA OPR recoverya (mean %) | Method 1623 OPR recovery (mean %) | CC-IFA MS recovery infectious oocysts b (mean %) | Method 1623 MS recovery total oocysts (mean %) |
|---------------|-----------------------------------|-------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------------------|
| Round 1       |                                   |                               |                                   |                                               |                                             |
| 1             | 61 66 47                           | 37 13                         |                                   |                                               |                                             |
| 2             | 50 24 71                           | 58 25                         |                                   |                                               |                                             |
| 3             | 56 65 68                           | 64 49                         |                                   |                                               |                                             |
| Round 2       |                                   |                               |                                   |                                               |                                             |
| 4             | 60 60 56                           | 31 9                           |                                   |                                               |                                             |
| 5             | 62 42 60                           | 27 33                         |                                   |                                               |                                             |
| 6             | 73 69 53                           | 49 46                         |                                   |                                               |                                             |
| Total mean   | 60 ± 11                           | 54 ± 18                       | 59 ± 9                           | 44 ± 16 | 29 ± 17 |

*Note.* CC-IFA: cell culture immunofluorescent assay; MS: matrix spike; OPR: ongoing precision and recovery.  
a Events 1–3 oocyst lot 4; events 4–6 oocyst lot 5.  
b Sample size per each of six events.  
c CC-IFA % recovery calculated using stock control as baseline infectivity.
DISCUSSION

The Cryptosporidium CC-IFA used in this study was based on previous work (e.g., Di Giovanni & LeChevallier, 2005, Johnson et al., 2012) and recent advancements described by King et al. (2012). Procedural improvements included bile stimulation of oocyst excystation, centrifugation of oocysts onto cell monolayers, and a low concentration of serum in the cell culture inoculation medium. Although these conditions were described previously by other authors, King et al. identified a highly successful combination of these steps, resulting in significantly higher rates of oocyst infectivity and the development of larger foci of infection. In particular, these modifications have greatly improved the cell culture infectivity for C. hominis. In earlier work, C. hominis oocyst stocks had an initial cell culture infectivity of $\leq 11\%$ (Johnson et al., 2012). In contrast, C. hominis controls had a mean $60\%$ infectivity in the present study. The improved CC-IFA conditions used in this study allowed us to evaluate method performance using 1–5 C. parvum and 3–10 C. hominis oocysts.

The capability to determine the infectivity of oocysts detected in the prefinished water leaving Hillview Reservoir is of importance to NYC. During 2011–2017, more than 95% of 341 Hillview Reservoir samples analyzed using Method 1623 tested negative for Cryptosporidium. Of the few positive samples, one sample contained two oocysts, while the remaining samples contained only a single oocyst. For follow-up monitoring when these infrequent, low-level detections occur, it would be beneficial to have an analytical tool that provides additional information about an oocyst’s ability to cause disease in humans. Since Method 1623 detects both infectious and noninfectious oocysts, including oocysts that have been inactivated by UV treatment and cannot cause illness, it likely overestimates risk. Such is the case for NYC as the water entering Hillview Reservoir has passed through a UV treatment process. The Hillview Reservoir is well-protected and essentially has no watershed. Although unlikely, it is possible that an oocyst derived from a small mammal or bird might enter the reservoir post-treatment. Because differentiating between UV-treated/ inactivated and nondisinfected oocysts is not possible with Method 1623, a method to determine the infectivity of the major human pathogenic species C. parvum and C. hominis, such as the version of the CC-IFA process studied here, provides the information needed to more accurately assess public health risk. Work by others has shown that CC-IFA assays similar to that used in the current study (including the use of HCT-8 cells and same IFA antibody) were capable of detecting less significant human pathogenic species, including C. meleagridis (Rochelle, Johnson, De Leon, & Di Giovanni, 2012; King, Fanok, Phillips, Swaffer, & Monis, 2015), C. cuniculus (Robinson et al., 2010; King et al., 2015), and C. ubiquitum (King et al., 2015). With the exception of rare, individual cases caused by typically animal-associated species and genotypes, this covers the species identified in almost all human cryptosporidiosis cases documented worldwide.

Equally important is the sensitivity of a method to detect low levels of the target Cryptosporidium species. By examining both C. parvum and C. hominis as target organisms, this study evaluated the success of detecting the Cryptosporidium species responsible for approximately 95% of human cryptosporidiosis cases. Moreover, by spiking low levels of oocysts, rather than the standard MS dose of 100 oocysts, the levels of oocysts more closely resembled those seen in the environment. This challenged the sensitivity of the method under realistic conditions and resulted in CC-IFA percent recoveries similar to control samples.

| No. of viable oocysts | CC-IFA infection control | CC-IFA trip control | CC-IFA matrix sample |
|-----------------------|--------------------------|---------------------|----------------------|
| C. parvum             |                          |                     |                      |
| 1                     | Pos. wells/total         | 6/10                | 4/10                 | 7/10                 |
| 3                     | Mean foci/pos. well      | 1.0 ± 0             | 1.4 ± 0.9            | 1.1 ± 0.4            |
| 5                     | Mean foci/pos. well      | 2.2 ± 0.8           | 2.1 ± 1.1            | 2.2 ± 1.0            |
| C. hominis            |                          |                     |                      |
| 3                     | Pos. wells/total         | 8/10                | 7/10                 | 8/10                 |
| 5                     | Mean foci/pos. well      | 1.8 ± 0.7           | 1.9 ± 1.2            | 2.3 ± 1.3            |
| 10                    | Mean foci/pos. well      | 4.9 ± 2.0           | 4.7 ± 1.9            | 4.8 ± 2.0            |

Note. CC-IFA: cell culture immunofluorescent assay; pos.: positive.

6 CONCLUSIONS

In this study, an improved CC-IFA method was evaluated to determine whether it could recover and detect precise
numbers of infectious C. parvum and C. hominis oocysts spiked into the NYC Hillview Reservoir water matrix. Similar recoveries of infectious oocysts by CC-IFA and total oocysts recovered with Method 1623 were obtained for C. parvum and C. hominis MS samples, demonstrating successful application of the CC-IFA to Hillview Reservoir samples. Moreover, the CC-IFA method performed well in MS trials, with 1–5 C. parvum or 3–10 C. hominis oocysts, demonstrating the ability to detect environmentally relevant numbers of infectious oocysts in the presence of the Hillview Reservoir matrix. Therefore, CC-IFA appears to be a promising tool to meet the needs of NYC and possibly other water utilities interested in refining Cryptosporidium risk based on oocyst infectivity.

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CONFLICT OF INTEREST

None.

REFERENCES

Bukhari, Z., Hargy, T. M., Bolton, J. R., Dussert, B., & Clancy, J. L. (1999). Medium-pressure UV for oocyst inactivation. *Journal AWWA*, 91(3), 86. https://doi.org/10.1002/j.1551-8833.1999.tb08602.x

Clancy, J. L. (Ed.). (2000). *Cryptosporidium viability and infectivity methods. Project 395*. Denver, CO: AWWA Research Foundation.

Di Giovanni, G. D., & LeChevallier, M. W. (2005). Quantitative PCR assessment of Cryptosporidium parvum cell culture infection. *Applied and Environmental Microbiology*, 71(3), 1495.

Johnson, A. M., Di Giovanni, G. D., & Rochelle, P. A. (2012). Comparison of assays for sensitive and reproducible detection of cell culture-infectious Cryptosporidium parvum and Cryptosporidium hominis in drinking water. *Applied and Environmental Microbiology*, 78(1), 156.

King, B., Fanok, S., Phillips, R., Swaffer, B., & Monis, P. (2015). Integrated Cryptosporidium assay to determine oocyst density, infectivity, and genotype for risk assessment of source and reuse water. *Applied and Environmental Microbiology*, 81(10), 3471.

King, B. J., Keegan, A. R., Phillips, R., Fanok, S., & Monis, P. T. (2012). Dissection of the hierarchy and synergism of the bile derived signal on Cryptosporidium parvum excystation and infectivity. *Parasitology*, 139(12), 1533.

Lalancette, C., Généreux, M., Mailly, J., Servais, P., Côté, C., Michaud, A., ... Prévost, M. (2012). Total and infectious Cryptosporidium oocyst and total Giardia cyst concentrations from distinct agricultural and urban contamination sources in Eastern Canada. *Journal of Water and Health*, 10(1), 147.

LeChevallier, M. W., Di Giovanni, G. D., Clancy, J. L., Bukhari, Z., Bukhari, S., Rosen, J. S., ... Frey, M. M. (2003). Comparison of Method 1623 and cell culture-PCR for detection of Cryptosporidium spp. in source waters. *Applied and Environmental Microbiology*, 69(2), 971.

Nichols, R. A., Connelly, L., Sullivan, C. B., & Smith, H. V. (2010). Identification of Cryptosporidium species and genotypes in Scottish raw and drinking waters during a one-year monitoring period. *Applied and Environmental Microbiology*, 76(17), 5977.

Ongerth, J. E. (2013). LT2 Cryptosporidium data: What do they tell us about Cryptosporidium in surface water in the United States? *Environmental Science & Technology*, 47(9), 4029.

Robinson, G., Wright, S., Elwin, K., Hadfield, S. J., Katzer, F., Bartley, P. M., ... Chalmers, R. M. (2010). Re-description of Cryptosporidium cuniculus Inman and Takeuchi, 1979 (Apicomplexa: Cryptosporidiidae): Morphology, biology and phylogeny. *International Journal of Parasitology*, 40(13), 1539.

Rochelle, P. A., Di Giovanni, G. D., Hoffman, R. M., Klonicki, P. T., McCuin, R. M., & Sturbauam, G. D. (2012). 9711 D Infectivity of Cryptosporidium in cell culture. In *Standard Methods for the Examination of Water and Wastewater* (22nd ed.). Washington, DC: APHA, AWWA, and WEF.

Rochelle, P. A., Johnson, A. M., De Leon, R., & Di Giovanni, G. D. (2012). Assessing the risk of infectious Cryptosporidium in drinking water. *Journal AWWA*, 104(5), E325. https://doi.org/10.5942/jawa.2012.104.0063

Rochelle, P. A., Johnson, A. M., & Di Giovanni, G. D. (2010). Detection of infectious Cryptosporidium in conventionally treated drinking water, *Project 302*. Denver, CO: AWWA Research Foundation.

Ruecker, N. J., Matsune, J. C., Wilkes, G., Lapen, D. R., Topp, E., Edge, T. A., ... Neumann, N. F. (2012). Molecular and phylogenetic approaches for assessing sources of Cryptosporidium contamination in water. *Water Research*, 46(16), 5135.

Sifuentes, L. Y., & Di Giovanni, G. D. (2007). Aged HCT-8 cell monolayers support Cryptosporidium parvum infection. *Applied and Environmental Microbiology*, 73(23), 7548.

U.S. Environmental Protection Agency. (2005). *Method 1623: Cryptosporidium and Giardia in water by filtration/IMS/FA*. Washington, DC: Author.

U.S. Environmental Protection Agency. (2012). *Method 1623.1: Cryptosporidi- um and Giardia in water by filtration/IMS/FA*. Washington, DC: Author.

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