Integrated *Cryptosporidium* Assay To Determine Oocyst Density, Infectivity, and Genotype for Risk Assessment of Source and Reuse Water

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Cryptosporidium continues to be problematic for the water industry, with risk assessments often indicating that treatment barriers may fail under extreme conditions. However, risk analyses have historically used oocyst densities and not considered either oocyst infectivity or species/genotype, which can result in an overestimation of risk if the oocysts are not human infective. We describe an integrated assay for determining oocyst density, infectivity, and genotype from a single-sample concentrate, an important advance that overcomes the need for processing multiple-grab samples or splitting sample concentrates for separate analyses. The assay incorporates an oocyst recovery control and is compatible with standard primary concentration techniques. Oocysts were purified from primary concentrates using immunomagnetic separation prior to processing by an infectivity assay. Plate-based cell culture was used to detect infectious foci, with a monolayer washing protocol developed to allow recovery and enumeration of oocysts. A simple DNA extraction protocol was developed to allow typing of any wells containing infectious *Cryptosporidium*. Water samples from a variety of source water and wastewater matrices, including a semirural catchment, wastewater, an aquifer recharge site, and storm water, were analyzed using the assay. Results demonstrate that the assay can reliably determine oocyst densities, infectivity, and genotype from single-grab samples for a variety of water matrices and emphasize the varying nature of *Cryptosporidium* risk extant throughout source waters and wastewaters. This assay should therefore enable a more comprehensive understanding of *Cryptosporidium* risk for different water sources, assisting in the selection of appropriate risk mitigation measures.

Cryptosporidium is ubiquitous in source waters and wastewaters, presenting a treatment challenge on account of its small size, resistance to chlorine disinfection, and the absence of more easily measured surrogates to allow treatment performance validation (1, 2). These characteristics make *Cryptosporidium* problematic for the water industry, and the ever-present threat from this pathogen requires sound characterization and management of risks, including validation and monitoring of critical control points (3–5). Removal of *Cryptosporidium* by treatment processes can be highly variable (6), so validation and monitoring of individual processes are important to ensure appropriate performance. Even for validated systems, treatment failure is possible under highly adverse conditions, and management of this risk may require significant capital expenditure to provide sufficient risk mitigation for extreme events. However, risk assessments frequently utilize historical monitoring data based on total oocyst numbers, not considering either oocyst infectivity or species/genotype, resulting in possible overestimation of risk.

Of the greater than 26 species or genotypes of *Cryptosporidium* that might be detected in the environment, only *C. parvum* and *C. hominis* commonly infect humans (7). Furthermore, not all oocysts excreted by an infected host are infectious, and those that are infectious oocysts can be rapidly inactivated by environmental conditions (8). *Cryptosporidium* species are commonly monitored in waters using standard detection methodologies (e.g., EPA Method 1622 and Method 1623). However, these methods provide no information on either the infectivity or identity of the detected oocyst, providing little information on the relative health risk posed to humans (9, 10).

Recently, intensive efforts have been made to examine source waters destined for potable consumption, as well as wastewaters

intended for reuse, using genotyping assays and, to a lesser extent, infectivity assays (9–13). The information from these assays provides an indication of the risk to human health (13). However, to better estimate risk, there is a requirement to understand oocyst density (number), infectivity, and species (13). While a number of assays are available to obtain such data, none are in a single-assay format, meaning that multiple-grab samples must be processed to conduct the different analyses, increasing analytical costs, or sample concentrates need to be split, compromising detection limits. The heterogeneous distribution and low numbers of oocysts in surface waters also complicate the use of multiple-grab samples, as oocysts can be present in one sample and absent in the next. These issues translate into higher costs to obtain data from all analyses, as well as potential variability in data quality when using multiple-grab samples.

To address a number of these shortcomings, Lalancette and

Received 20 January 2015 Accepted 6 March 2015

Accepted manuscript posted online 13 March 2015

Citation 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. *Appl Environ Microbiol* 81:3471–3481. doi:10.1128/AEM.00163-15.

Editor: D. W. Schaffner

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00163-15>.

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FIG 1 Overview of the workflow processes of the integrated assay. The assay is described as two discrete stages, front end processing and back end processing. Front end processing involves the concentration of oocysts from samples and their capture onto IMS beads, and the back end processing, which is the novel part of the assay, refers to the elution and subsequent treatment of oocysts from the IMS (immunomagnetic separation) beads in order to determine densities, infectivity, and genotype from a single sample.

colleagues developed a dual-direct-detection assay to provide information on the fraction of infectious oocysts from a water sample (10, 13). To achieve this, they combined a cell culture immunofluorescence infectivity assay with a direct count of oocysts on the host cell monolayer as well as from washes off the monolayer using a filtration technique to capture oocysts. This prompted us to adapt an optimized infectivity assay (14, 15) to not only include a total oocyst count for calculating the percentage of infectious oocysts but also incorporate recovery controls to account for oocyst losses at different process steps. Furthermore, we developed an extraction step to genotype *Cryptosporidium* life cycle stages present in infectious foci, and we assessed the feasibility of genotyping oocysts remaining on monolayers that did not contain any infectious foci.

To determine the performance of our integrated assay for measuring oocyst densities and infectivity and determining genotype,

we applied it to water samples collected from a series of rain events in an Adelaide Hills semirural/agricultural catchment, various sampling points at three wastewater treatment plants, an influent aquifer recharge site, and inlets for two storm water collection schemes. Here we present results from these activities demonstrating that this integrated assay is capable of determining oocyst densities, infectivity, and genotype from single-grab samples across a variety of water matrices, thereby providing a more comprehensive understanding of *Cryptosporidium* risk.

MATERIALS AND METHODS

Integrated assay overview. Figure 1 presents an overview of the work flow for the integrated assay to generate information on oocyst densities, infectivity, and genotype from water samples. The assay is described as two discrete stages, front end processing and back end processing. Front end processing involves primary concentration of oocysts from a water sam-

ple, followed by secondary concentration and purification of oocysts by immunomagnetic separation (IMS). The back end processing, which is the novel part of the assay and focus of this paper, refers to the elution of oocysts from the IMS beads and the subsequent steps required to determine oocyst density, infectivity, and genotype from a single sample.

Source of control *Cryptosporidium* oocysts. The *C. parvum* cattle isolate (strain Iowa) used in these studies was purchased from BTF (Sydney, Australia). Oocysts were enumerated by dilution in phosphate-buffered saline, followed by staining with EasyStain, filtration onto 13-mm black polycarbonate membranes (0.8- μ m pore size), and enumeration using fluorescence microscopy (described in detail below). On receipt, the infectivity of each oocyst batch was determined using the focus detection method (14, 15). All lots were stored at 4°C and used within 16 weeks of receipt. ColorSeed (BTF, Sydney, Australia), which contains 100 Texas Red-labeled gamma-inactivated *C. parvum* oocysts, was used as an internal oocyst recovery control.

In vitro culturing of the HCT-8 cell line. Cells from the HCT-8 cell line (ATCC CCL-244; human ileocecal colorectal adenocarcinoma) were maintained in 25-cm² flasks with regular subculturing (3 times per week) in RPMI 1640 growth medium with L-glutamine and supplemented with 15 mM HEPES buffer, 100,000 U/liter penicillin G, 0.1 g/liter streptomycin, and 10% fetal calf serum adjusted to pH 7.4. HCT-8 cells were incubated at 37°C in a humidified CO₂ (5% [vol/vol]) incubator until confluent. All cell culture reagents were purchased from Sigma-Aldrich, Sydney, Australia. Confluent monolayers were used to inoculate 48-well plates (Nunclo Δ Surface) at approximately 4×10^5 cells/well and grown until confluent over a 48-h incubation period (humidified at 37°C with 5% [vol/vol] CO₂) before infection.

Concentration and isolation of environmental oocysts from source waters and wastewaters. All water samples were lodged with the Australian Water Quality Centre (a laboratory accredited for *Cryptosporidium* analysis with the Australian National Association of Testing Authorities) for front end processing. Raw sewage samples were subsampled (250 ml to 1,000 ml) and diluted in either 5 liters or 10 liters of reverse osmosis water before primary concentration. Either 5-liter or 10-liter volumes were processed for source waters or treated wastewaters. Samples (either diluted or neat) were seeded with 100 ColorSeed oocysts immediately prior to concentration, according to the manufacturer's recommendation for adding ColorSeed to samples. ColorSeed is an internal recovery control allowing the verification of method performance and determination of oocyst recovery rates for each sample processed.

Primary concentration of water samples used the calcium carbonate precipitation method as previously described (16). The resulting sample pellet was weighed prior to processing, and the secondary concentration step was carried out using a *Cryptosporidium* IMS kit (Dyna, Mulgrave, Australia) according to the manufacturer's instructions. A maximum of 0.5 g of sample pellet was processed per IMS tube, with up to 2 IMS tubes used per sample. IMS tubes from the same sample were processed separately for all subsequent steps. Resulting IMS pellets were washed once with phosphate-buffered saline (PBS) before oocysts were dissociated from the beads by incubation in 50 μ l 0.1 N HCl for 10 min at room temperature. The supernatant (containing dissociated oocysts) was then transferred to tubes containing 5 μ l of 1 M NaOH to allow for neutralization. This step is critical because prolonged exposure to acidic conditions can cause premature excystation of oocysts, adversely affecting the infectivity assay. The IMS beads were further washed using 945 μ l of acidified water trypsin solution (acidified water [pH 2.4] and 0.025% [wt/vol] trypsin) to remove any enmeshed oocysts, with the wash solution combined with the supernatant. The supernatant containing oocysts in acidified water was then immediately taken through the excystation pretreatment protocol (described below) before subsequent infection of the cell line. For each IMS pellet, eluted oocysts were inoculated into separate cell culture wells.

Excystation pretreatment. A modified excystation pretreatment technique for *C. parvum* oocysts (14, 15) was used to delay excystation until

the oocysts were in contact with the host cell monolayer. The acidified water/trypsin oocyst suspension was incubated for 20 min at 30°C and then centrifuged for 10 min at 1,800 relative centrifugal force (RCF) before careful aspiration of 900 μ l of the supernatant. The oocyst pellet was washed by two rounds of resuspension in 900 μ l of infection medium, centrifugation for 10 min at 1,800 RCF, and careful aspiration of 900 μ l of infection medium. The oocyst pellet was then resuspended in 900 μ l of fresh infection medium prewarmed to 37°C and inoculated onto cell monolayers that had had the growth medium previously removed. The infection medium consisted of RPMI 1640 medium with L-glutamine, 15 mM HEPES buffer, sodium bicarbonate (2 g/liter), glucose (1.0 g/liter), bovine bile (0.2 g/liter), folic acid (250 μ g/liter), 4-aminobenzoic acid (1 mg/liter), calcium pantothenate (50 μ g/liter), ascorbic acid (8,750 μ g/liter), penicillin G (100,000 U/liter), streptomycin (100 mg/liter), lincomycin (40 mg/liter), and gentamicin (50 mg/liter). All cell culture reagents were purchased from Sigma-Aldrich, Sydney, Australia. The infection medium was at pH 7.4 $\pm$ 0.1.

Focus detection method. Oocysts were applied to the cell culture monolayers after excystation pretreatment and centrifuged onto the monolayer at 408 RCF for 5 min using an Eppendorf (model 5804) centrifuge plate spinner. This step ensured that oocysts were in contact with the cell monolayer prior to excystation. Inoculated monolayers were incubated for 4 h in a humidified incubator at 37°C with 5% (vol/vol) CO₂ to allow for attachment/invasion to occur. After this initial incubation period, oocysts were recovered from the monolayer (described below). Wells were then replenished with 1 ml of prewarmed infection medium (37°C) and returned to the incubator for a further 44 h. Cell monolayers were then fixed with methanol and stained with Sporo-Glo (a sporozoite polyclonal antibody that binds strongly to merozoites and all other intracellular reproductive stages [Waterborne Inc., New Orleans, LA, USA]), and infectious foci were enumerated as previously described (14) using an inverted Olympus IX51 fluorescence microscope.

Recovery and enumeration of oocysts from infected monolayers. In order to incorporate a total oocyst count into the assay, an oocyst recovery technique was trialed for samples collected from a variety of water matrices and sources. After the 4-h incubation step to allow for attachment/invasion (described above), 700 μ l of the infection medium within the cell culture well was vigorously but carefully repipetted up and down (5 to 10 times), expelling the medium with each action against the side wall of the culture plate so that the force of the expulsion of the medium did not directly contact and damage the cell monolayer but facilitated oocyst removal from the monolayer and resuspension back into the medium. The infection medium (700 μ l) was then removed from each well into separate clean tubes. The remaining infection medium (300 μ l) was then removed and combined with the 700- μ l volume already recovered. Monolayers were replenished with 1 ml of fresh infection medium (prewarmed to 37°C) and returned to the incubator as described above. The medium collected from each individual cell monolayer was then stained and enumerated as described below.

Filtration counting of oocysts. One-milliliter volumes of oocyst suspensions were stained with 30 μ l EasyStain (a fluorescein isothiocyanate [FITC]) monoclonal antibody highly specific to the oocyst wall; BTF, Sydney, Australia). Samples were incubated for 1 h in the dark at room temperature and then filtered through 13-mm black polycarbonate membranes with a nominal pore size of 0.8 μ m (Rowe Scientific, Lonsdale, South Australia) on a vacuum manifold system fitted with Swinnex filter holders (Millipore, Kilsyth, Australia) at a vacuum pressure of 200 mbar. Membranes were mounted on glass microscope slides with 7 μ l of mounting medium (BTF, Sydney, Australia) and sealed with a coverslip. Entire membranes were scanned, and all oocysts (ColorSeed and environmental) were counted by fluorescence microscopy using either Olympus (Nottingham, Australia) BX40 or BX60 microscopes. Using this methodology, the oocyst count is considered presumptive because in most cases internal contents are not present to verify that green fluorescent objects are *Cryp-*

cryptosporidium oocysts and in some cases the shape/size can be atypical due to deformation of empty oocysts under vacuum pressure.

Recovery and extraction of infected cell culture monolayer. In order to further characterize *Cryptosporidium* life cycle stages present in the monolayer, cells were recovered by adding 200 μ l of 0.25% trypsin-EDTA solution (product number T4049; Sigma, Sydney, Australia) to each fixed cell culture well and incubating the wells at 37°C for 10 min. Using non-filter tips, the sticky pellet was carefully removed, transferred to a 1.5-ml microcentrifuge tube, and centrifuged for 10 min at 20,000 RCF. The supernatant was removed, and the pellet was washed with 200 μ l of 1 $\times$ PCR buffer II (Applied Biosystems, Life Technologies, Mulgrave, Victoria) and centrifuged for 10 min at 20,000 RCF. The supernatant was removed, and 50 μ l 1 $\times$ PCR buffer II was added before incubation at 100°C for 10 min. Samples were centrifuged for 10 min at 20,000 RCF, after which the crude lysate containing the extracted DNA was stored at -30°C prior to PCR analysis.

PCR analysis and genotyping. Real-time PCR was performed on a Rotor-Gene 6000 HRM (Qiagen, Chadstone, Australia) for the 18S rRNA, *gp60*, and *lib13* loci. The PCR conditions and primers and probes utilized were as previously described for *gp60* and 18S rRNA (17) and *lib13* (18). Crude lysates containing DNA from extracted cell monolayers were used as the templates for PCR analysis. Reaction volumes were 25 μ l. For nested PCRs, first-round reaction volumes were diluted 1/100 before use in the second-round PCR. Controls for *C. parvum* were prepared as previously described (19). Controls for *C. hominis* were originally collected from human clinical specimens under University of South Australia human ethics approval number 0000021356 and were prepared as previously described (17). Control genomic DNA samples of other *Cryptosporidium* species/genotypes were obtained from the *Cryptosporidium* Reference Unit (Public Health Wales, Swansea, United Kingdom), care of Kirsten Elwin, and from Murdoch University (Perth, WA, Australia), care of Una Ryan. DNA melt curve analysis, sequencing, and phylogenetic analysis for species identification were performed as previously described (17).

Excystation pretreatment and monolayer recovery controls. To determine oocyst losses during the excystation pretreatment and to calculate oocyst recoveries from cell monolayers, aliquots of 100 *C. parvum* cattle isolate (strain Iowa) oocysts in 100 μ l of PBS were prepared and are referred to here as “shots.” Two triplicate shots were used in each round of sample processing as batch controls. The first triplicate set was used to determine losses from the excystation pretreatment process. These oocysts were taken through the entire excystation protocol (described above), but instead of inoculating oocysts onto monolayers, controls were stained with antibody and enumerated. The second set of triplicate shots was used as a monolayer infection and oocyst recovery control. These oocysts were taken through the entire excystation protocol and inoculated onto cell monolayers before being recovered as described above. The oocyst counts from these controls allow an estimation of oocyst losses on the monolayer (required to calculate the number of oocysts applied to the monolayer). For each experimental batch, the foci from the positive batch controls (second set of triplicate shots applied to the monolayer) were enumerated. This provides verification that the cell monolayer is still capable of supporting infection.

Collection of water samples from an agricultural/rural catchment. Water samples were collected from five watersheds within an Adelaide Hills (South Australia) catchment area dominated by livestock grazing but also with some areas of native vegetation. Twelve rainfall-runoff events occurred (approximately 370 mm cumulative rainfall) from July 2013 through August 2013, of which 8 events were sampled and analyzed. For watersheds 2, 3, 5, and 7, 10 10-liter samples were collected from each sampling point for each rainfall event. These samples were then mixed to provide a 100-liter composite sample, with quadruplicate 10-liter aliquots of the composite sample then processed as described above. Watershed 4 samples were collected for only 2 rainfall events. In total, 136 individual samples (34 composite samples) were processed and analyzed using the single-format assay. The approach to make composite samples was un-

dertaken to ensure that the composite was representative of the event conditions over the duration of the event hydrograph and to minimize sample variability within a location in a given event.

Collection of samples from wastewater treatment plants. Three wastewater treatment plants (WWTPs) located in Victoria, representing differing treatment processes, were chosen for evaluating the integrated assay as part of a larger project investigating the fate of *Cryptosporidium* through the wastewater treatment train. Plant A processes around one-half of the sewage for the city of Melbourne and produces approximately 40 billion liters of recycled water a year. It uses a series of anaerobic and aerobic ponds and lagoons. Plant B services over 20,000 residential and business properties and treats close to 13 million liters of sewage a day. It utilizes sequencing batch reactors, tertiary cloth filtration, UV disinfection, and reverse osmosis to supply up to 2.5 billion liters per year of recycled water. Plant C serves a township and treats approximately 12.5 million liters of sewage a day. This plant uses the activated-sludge processes prior to chlorination, dechlorination, and discharge. Samples representing raw, primary, secondary, and tertiary treated effluent were collected from various points along the treatment trains from January 2013 to October 2013. Samples were collected in either duplicate (plant A) or triplicate (plants B and C) from each sampling point and transported overnight by courier to the laboratory for processing.

Collection of catchment samples from an aquifer recharge site. The aquifer recharge site was located near a regional town in South Australia (250 km southeast of Adelaide). Twenty 10-liter samples were collected on 28 August 2013, when water was entering runaway holes to recharge the aquifer. The source water was from a creek receiving runoff from a mixed-use catchment comprising agricultural and urban inputs. Upon collection, samples were immediately transported to the laboratory for processing.

Collection of storm water samples. The storm water harvesting sites were within the Adelaide metropolitan area. Site A is located approximately 8 km to the southwest of Adelaide and has a predominantly residential catchment, with potential upstream inputs from a nonurbanized reserve and rural areas in the Adelaide Hills. This reuse scheme has been designed to harvest up to 350 Ml of storm water per year. Site B is located approximately 15 km north-northwest of Adelaide and collects storm water from a catchment with industrial and residential inputs. Single 10-liter samples were collected from four rainfall-runoff events (>5 mm rainfall) from each site from May 2013 through September 2013.

Calculation of oocyst densities. *Cryptosporidium* counts were adjusted to account for recovery rate, sample volume, and the amount of pellet processed and then expressed as oocyst density (presumptive number of oocysts per 10 liters). The formula to convert the observed count to a presumptive count per 10 liters is as follows: Oocyst density/10 liters = (Enumerated oocysts/*R*) $\times$ *S* $\times$ *P*, where *R* is the recovery fraction, *S* is the sample volume multiplier, and *P* is the pellet volume multiplier. *R*, *S*, and *P* are calculated as follows. *R* = (ColorSeed enumerated $\times$ *P*)/ColorSeed added; *S* = 10 liters/Sample volume processed (in liters); *P* = Pellet size/Pellet processed, where pellet size and pellet processed are in grams.

Calculation of the infectious oocyst fraction. The formula below was used to calculate the infectious oocyst fraction (as a percentage) from a single sample or an entire event: Infectious oocyst fraction = (Foci enumerated/Oocysts inoculated onto culture) $\times$ 100, where Oocysts inoculated onto culture = Enumerated oocysts/Plate recovery, and Plate recovery = Mean of plate recovery controls/Mean of preexcystation controls.

RESULTS

Oocyst recovery and infectivity for excystation pretreatment and monolayer batch controls. Batch controls were developed to determine (i) oocyst losses during excystation pretreatment sample processing (and conversely the number of control oocysts applied to the monolayer), (ii) the efficiency of the plate washes to recover oocysts from the monolayer, and (iii) that the infectivity assay has performed adequately for any given batch of reagents,

TABLE 1 Oocyst batch control recoveries and infectivities for the integrated assay from the Adelaide Hills agricultural/rural catchment^a

Experimental setup batch no.	Mean no. $\pm$ SD of oocysts recovered:		Plate recovery (%)	Mean no. $\pm$ SD of foci detected	Infectious fraction (%)
	After preexcystation	From monolayers			
1	106 $\pm$ 8	93 $\pm$ 8	88	53 $\pm$ 2	50
2	87 $\pm$ 3	79 $\pm$ 8	90	47 $\pm$ 8	54
3	90 $\pm$ 5	110 $\pm$ 11.5	122	61 $\pm$ 9	68
4	75 $\pm$ 19	60 $\pm$ 12	80	43 $\pm$ 8	57
5	89 $\pm$ 12	78 $\pm$ 6.5	88	38 $\pm$ 7	43
6	100 $\pm$ 13	75 $\pm$ 4	75	33 $\pm$ 2	33
7	94 $\pm$ 4	86 $\pm$ 4	91	42 $\pm$ 6	45
8	76 $\pm$ 3	65 $\pm$ 16	86	31 $\pm$ 13.5	40
9	92 $\pm$ 11	78 $\pm$ 8	85	33 $\pm$ 7	36
10	90 $\pm$ 2	80 $\pm$ 2	89	39 $\pm$ 4	43
11	80 $\pm$ 6	75 $\pm$ 6	94	35 $\pm$ 7	44
Mean	89 $\pm$ 9		90 $\pm$ 12		47 $\pm$ 10

^a The standard deviations of the means are from 3 batch controls.

cells, and control live oocysts. The percent plate recovery efficiency, calculated using the batch controls, is critical for correcting the sample oocyst count following recovery from the monolayer to estimate the number of oocysts from a sample that have been inoculated onto cell culture.

Table 1 presents data for these controls from 11 individual batch runs conducted to analyze samples collected over 8 rainfall events from an Adelaide Hills catchment. For the catchment samples, a mean recovery of 89% $\pm$ 9% (standard deviation) was achieved for the excystation pretreatment step, while a mean oocyst plate recovery of 90% $\pm$ 12% was achieved for those oocysts inoculated onto plates. Batch 3 was the only batch for which there was a reversal in the rank of the means between the two batch controls, suggesting some minor variation in the number of oocysts in the shots used as the batch controls. This variation appeared to be atypical and was present only for batch 3, which also had a higher number of infectious foci detected, while the other batches had similar plate recoveries and similar numbers of foci detected. The mean infectious oocyst fraction was 47% $\pm$ 10% for the 11 sets of batch controls.

ColorSeed recoveries from surface water and wastewater. ColorSeed, an internal oocyst calibration standard containing 100 Texas Red-labeled inactivated *Cryptosporidium* oocysts, was incorporated into the integrated assay and applied across a variety of water matrices to assess oocyst recoveries for the entire workflow (including both front end and back end processing), as well as to facilitate the calculation of oocyst densities. Table 2 presents the ColorSeed recoveries for 34 composite samples (each sample analyzed in quadruplicate) collected during 8 rain events from five watersheds within the Adelaide Hills catchment area. A mean ColorSeed recovery of 38% $\pm$ 7% was achieved for these samples. Of the 136 individual 10-liter samples processed in this catchment study, only 4 samples returned unacceptable recoveries (<10%). These four samples were all derived from one composite sample collected from watershed 3 during rain event 3.

Table 3 presents the ColorSeed recoveries for samples collected across 6 rounds of sampling from 7 sampling points representing raw, primary, and secondary treated effluent from plant A. The mean ColorSeed recovery was 40% $\pm$ 14% for these effluent sources. Of 84 samples, only 1 returned an unacceptable recovery (<10%). Furthermore, water samples representing an influent

aquifer recharge site (see Table S1 in the supplemental material) and inlets to two storm water collection schemes (see Table S2 in the supplemental material) were also analyzed using the integrated assay, with average recoveries of 37% $\pm$ 9% (n = 20) and 41% $\pm$ 12% (n = 8), respectively.

Oocyst density, infectivity, and genotype data for an agricultural/rural catchment. Oocyst density data were calculated for samples collected over 8 rain events from 5 watersheds within an agricultural/rural catchment (Fig. 2). Environmental oocysts were detected in all but 8 samples. Of these, 4 were replicate composite samples from watershed 5 rain event 7, with a mean ColorSeed recovery of 46%. The other 4 samples were replicate composite samples from watershed 3 rain event 3 and were deemed to be invalid due to low (<10%) ColorSeed recoveries. Excluding the invalid samples, the recovery rates were consistent across events and between watersheds (Table 2). While recovery rates were comparable across sites, there was some variation between sites in terms of oocyst densities. Watershed 2 showed the highest oocyst

TABLE 2 ColorSeed recoveries for samples analyzed from the Adelaide Hills agricultural/rural catchment^a

Rain event no.	% ColorSeed recovery $\pm$ SD from watershed no.:					Avg % rain event recovery $\pm$ SD
	2	3	4	5	7	
1	43 $\pm$ 14	44 $\pm$ 7	32 $\pm$ 14	37 $\pm$ 4	35 $\pm$ 5	38 $\pm$ 6
2	40 $\pm$ 20	45 $\pm$ 10	60 $\pm$ 25	44 $\pm$ 14	45 $\pm$ 16	47 $\pm$ 8
3	27 $\pm$ 8	1 $\pm$ 1 ^c	Δ^b	43 $\pm$ 5	35 $\pm$ 13	35 $\pm$ 8
4	37 $\pm$ 7	36 $\pm$ 6	Δ	42 $\pm$ 7	30 $\pm$ 5	36 $\pm$ 5
5	31 $\pm$ 6	36 $\pm$ 9	Δ	35 $\pm$ 2	23 $\pm$ 11	31 $\pm$ 6
6	33 $\pm$ 7	40 $\pm$ 11	Δ	37 $\pm$ 4	31 $\pm$ 9	35 $\pm$ 4
7	36 $\pm$ 7	52 $\pm$ 5	Δ	46 $\pm$ 3	37 $\pm$ 7	43 $\pm$ 8
8	29 $\pm$ 10	43 $\pm$ 7	Δ	38 $\pm$ 8	30 $\pm$ 5	35 $\pm$ 7
Avg watershed recovery	35 $\pm$ 6	42 $\pm$ 6	46 $\pm$ 20	40 $\pm$ 4	33 $\pm$ 6	

^a The standard deviations of the means are from 4 samples derived from a single composite sample.

^b Δ , no samples were collected for watershed 4 for rain events 3 to 8.

^c The mean recovery for watershed 3 from rain event 3 was not included in calculations for either average watershed recovery or average rain event recovery 4, as all sample replicates returned an invalid result due to low ColorSeed recoveries (<10%).

TABLE 3 ColorSeed recoveries for samples^a analyzed from various sampling points along a wastewater treatment plant (WWTP A)

Sampling point	% ColorSeed recovery ± SD from round no.:						Avg % recovery ± SD
	1	2	3	4	5	6	
Raw sewage	37 ± 2	19 ^b	57 ± 19	49 ± 1	64 ± 8	43 ± 2	45 ± 16
Anaerobic pot 1	32 ± 6	20 ± 7	35 ± 4	46 ± 7	60 ± 8	53 ± 11	41 ± 15
Anaerobic pot 2	62 ± 6	38 ± 6	39 ± 1	55 ± 6	63 ± 1	54 ± 6	52 ± 11
Clarifier	39 ± 4	43 ± 5	37 ± 1	46 ± 22	55 ± 12	24 ± 0	41 ± 10
Lagoon 2	48 ± 0	21 ± 16	31 ± 18	29 ± 3	54 ± 12	17 ± 1	33 ± 15
Lagoon 4	54 ± 23	33 ± 3	33 ± 4	28 ± 9	34 ± 20	33 ± 13	36 ± 9
Lagoon 6	40 ± 0	25 ± 6	35 ± 10	26 ± 7	32 ± 1	31 ± 17	32 ± 6

^a The standard deviations of the means from each round are derived from 2 individual grab samples.

^b Recovery rate is from an individual grab sample, as the duplicate was an invalid result.

densities, with 6 of 8 events exceeding 100 oocysts/10 liters, including event 2 with numbers exceeding 1,000 oocysts/10 liters. In comparison, the other watersheds generally had <100 oocysts/10 liters, with one-half of the events from watershed 7 having <10 oocysts/10 liters.

Interestingly, most of the *Cryptosporidium* oocysts detected in watershed 2 stained less brightly with the EasyStain antibody and were approximately 3.5 μm in size, which is outside the typical size range of 4 to 6 μm for the majority of intestinal *Cryptosporidium* species. Some small oocysts were also noted at other sites, but the

FIG 2 Oocyst density data for samples collected over 8 rain events across five watersheds within an Adelaide Hills catchment. The standard deviations of the means are from 4 samples derived from a single composite sample. No samples were collected for watershed 4 for rain events 3 to 8 (Δ). Watershed 3 event 3 returned an invalid result due to low ColorSeed recoveries (<10%).

TABLE 4 Characterization of infective foci detected from the Adelaide Hills agricultural/rural catchment^a

Rain event no.	Watershed no.	GP60 locus amplification	GP60 locus designation	Phylogenetic placement
2	2	Negative	NA ^b	<i>C. ryanae</i>
2	2	Negative	NA	<i>C. ryanae</i>
2	2	Negative	NA	<i>C. ryanae</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
2	4	Positive	<i>C. cuniculus</i>	<i>C. cuniculus</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
2	4	Negative	NA	<i>C. ubiquitum</i>
7	7	Negative	NA	Rat genotype

^a 18S amplification was positive for all infective foci.^b NA, not applicable.

majority of the *Cryptosporidium* oocysts detected in watersheds 3, 4, 5, and 7 were approximately 4.5 μm in size.

In total, 2,590 oocysts were enumerated from the 136 samples processed. Using the batch controls incorporated into the assay, 2,886 oocysts were calculated to have been inoculated onto cell cultures. A total of 11 infectious foci were detected, representing an infectious oocyst fraction of 0.4% for the combined results for oocysts analyzed from all of the watersheds within this catchment. However, 7 of the 11 foci detected were from a single location and event (Table 4), representing an infectious fraction of 2.7% for this site/event.

Those foci detected were identified by PCR amplification and DNA sequence analysis of regions of the 18S rRNA and *gp60* loci (Table 4). The species for 6 of the 11 foci detected were determined to be *C. ubiquitum*, 3 were *C. ryanae*, and the remaining ones were *C. cuniculus* and Rat genotype. *C. ryanae*, which has a mean size of 3.2 μm by 3.7 μm , was the only infectious species detected at watershed 2 (Table 4).

Selected samples that did not form infectious foci but represented a range of oocyst numbers inoculated onto culture were chosen for further analysis to determine if the oocysts remaining on cell monolayers after washing could be detected by PCR and genotyped (Table 5). Samples from rain event 3 had 44 or more

TABLE 5 Characterization of noninfectious oocysts from the Adelaide Hills agricultural/rural catchment^a

Rain event no.	Watershed	No. of oocysts inoculated onto culture	Plate recovery (%)	18S PCR	Species
3	2	104	75	Positive	<i>C. ryanae</i>
3	2	67	75	Positive	<i>C. ryanae</i>
3	2	114	75	Positive	<i>C. bovis</i>
3	2	96	75	Positive	<i>C. bovis</i>
3	2	44	75	Positive	<i>C. bovis</i>
1	2	27	90	Negative	NA ^b
1	2	33	90	Negative	NA
1	2	19	90	Negative	NA
1	2	24	90	Negative	NA
1	2	12	90	Negative	NA
1	2	21	90	Negative	NA

^a *gp60* PCR was negative for all noninfectious oocytes.^b NA, not applicable.

oocysts inoculated onto culture, and all were positive for the 18S rRNA nested PCR. These samples had an average plate recovery of 75%, so the minimum number of oocysts remaining on the monolayer for these samples would be 11. These oocysts were determined to be *C. bovis* or *C. ryanae*. Samples from rain event 1 had 33 or fewer oocysts inoculated onto monolayers and were negative for the 18S rRNA and *gp60* nested PCRs. These samples had plate recovery rates of 90%, representing 3 or fewer oocysts remaining on the monolayer for PCR detection.

Oocyst density, infectivity, and genotype data for samples collected from three wastewater treatment trains. Oocyst density and infectivity data were calculated for samples collected for a single round of sampling from 3 wastewater treatment plants (Fig. 3). Sample collection coincided with a significant increase in the number of cryptosporidiosis notifications for the geographical locations serviced by each of the WWTPs, which was reflected by a high oocyst density (approximately 10^5 oocysts/10 liters) within the raw sewage for all three plants. Density data generated from replicate grab samples showed that the method was reproducible, with little variation between replicates (Fig. 3). Based on the density data, oocyst removals across the treatment processes for plant A, plant B, and plant C were estimated to be 2.94 $\log_{10}$, 1.12 $\log_{10}$, and 1.95 $\log_{10}$, respectively.

Mean oocyst infectivity rates in the raw sewage for plants A, B, and C were 35% $\pm$ 0.6%, 52% $\pm$ 7%, and 50% $\pm$ 11%, respectively. Notably, in addition to oocyst removal across the treatment train, a decrease in oocyst infectivity was evident by the end of the treatment trains for both plant A and plant B. For plant A, a 1.5- $\log_{10}$ oocyst inactivation occurred by the end of lagoon 2, with at least a further 2- $\log_{10}$ oocyst inactivation by the end of lagoon 4, where no infectious oocysts were detected. For this round of sampling, the cumulative *Cryptosporidium* reduction for plant A incorporating both removal and inactivation was greater than 4.44 $\log_{10}$ (from raw sewage to lagoon 4).

For plant B, secondary treatment did not reduce oocyst infectivity compared with raw sewage. The secondary effluent had approximately 3,000 oocysts/10 liters, 52% of which were infectious, posing a significant challenge to the UV plant. However, no infectious oocysts were detected in the effluent after UV disinfection, representing an inactivation of greater than 3.24 $\log_{10}$. Combination of removal and inactivation data for plant B resulted in an overall reduction of >4.36 $\log_{10}$.

For plant C, there did not appear to be any decrease in infectivity of oocysts following activated sludge treatment and clarification, with physical removal following clarification being the main mechanism for reducing oocyst density.

Foci from selected sampling points from plants A, B, and C were analyzed using a *gp60* nested PCR combined with DNA melting curve analysis for species identification and *C. parvum* and *C. hominis* TaqMan PCR assays targeting *lib13*. The results (see Table S3 in the supplemental material) suggest that *C. hominis* was the dominant *Cryptosporidium* species present in these samples, although *C. parvum* was detected in a sample from plant A. Based on the DNA melting curve analysis, there was also evidence for the presence of another *gp60* amplicon, possibly *C. parvum*, in another plant A sample and plant B sample.

Oocyst density, infectivity, and genotype data from an aquifer recharge site and for samples collected from two storm water collection schemes. Of the 20 grab samples collected at an aquifer recharge site, only 1 sample was positive for a single noninfectious

FIG 3 Oocyst density and infectivity data collected over a single round from three wastewater treatment plants. Samples represent raw, primary, secondary, and tertiary treated effluent and were collected from various points along the treatment trains. Samples were collected in duplicate for each sampling point for treatment plant A and in triplicate for treatment plants B and C. The LLP Auto sampling point for treatment plant B is equivalent to a clarifier sample.

environmental oocyst (see Table S1 in the supplemental material). In contrast, oocysts were detected in the majority of storm water samples, with totals of 183 and 156 oocysts and mean oocyst densities of 46 ± 26 oocysts/10 liters and 39 ± 42 oocysts/10 liters detected at site A and site B, respectively (see Table S2 in the supplemental material). Three infective foci were detected in the first round of sampling, 2 at site A and 1 at site B. Based on the number of oocysts calculated to have been inoculated onto culture for the first round of sampling, the infectious fraction for this event was determined to be 13% for site A and 3.4% for site B. The infectious sample from site A showed a sequence that was most similar to *Cryptosporidium* sequence with GenBank accession no. EU546850, isolated from a brush tail possum (20), while the infectious sample from site B was determined to be *C. baileyi*.

DISCUSSION

Risk analyses for *Cryptosporidium* in source waters have frequently been undertaken using total oocyst numbers but only occasionally considered species presence and less commonly species prevalence or oocyst infectivity (21–23). In part, this has been due to the absence of an assay capable of generating information from a single sample for all of these parameters. To address this, we modified a highly sensitive infectivity assay capable of detecting a single infectious oocyst (14), converting the assay format from a Labtek slide culture system to a plate-based assay and developing a simple DNA extraction method to allow PCR-based identification of any infectious foci. This modified assay further built upon the work of Lalancette et al. (10, 13), incorporating a variety of

oocyst recovery controls to allow estimates of the number of oocysts applied to cell culture for determination of infectivity ratios and of the number of oocysts in the original water sample. This new assay was evaluated using a variety of water matrices.

In order to enumerate oocysts from a water sample, we utilized a technique involving resuspension and recovery of oocysts from the cell monolayer. ColorSeed oocysts were included as an internal control for every sample, allowing the determination of oocyst recovery rates across the entire process (from primary concentration to recovery of oocysts from the cell monolayer). Batch controls were also used for each plate to allow the determination of oocyst recoveries following the excystation pretreatment and resuspension from the monolayers. The latter control was critical for estimating the number of oocysts applied to the monolayer (required for calculating percent infectivity) and demonstrated only minor losses through these processing steps. The incorporation of an oocyst count with a recovery rate into the infectivity assay represents a substantial advance over processing replicate grab samples or splitting water sample concentrates into multiple aliquots for separate analyses. This is of particular importance for samples with low oocyst densities, where replicate grab samples or concentrated aliquots may not contain oocysts, affecting the quality of the information generated.

The oocyst recovery process described herein is an improvement over the need to enumerate oocysts still left on the monolayer in addition to those captured from washes. Enumerating oocysts on monolayers requires the use of antibodies with different fluorescent labels to allow discrimination of oocysts from the life cycle stages in infectious foci (10, 13). Commercial sources of anti-oocyst and anti-life cycle stage antibodies commonly use fluorescein derivatives as the label, so detection of oocysts on the monolayer would require the use of custom-labeled antibodies. Furthermore, the choice of alternative fluorophores is limited. There is a practical limit to the number of filters used on microscopes for the visualization of different fluorophores, and the use of red fluorophores is not compatible with the incorporation of ColorSeed (Texas Red labeled) oocysts into such an assay. Our capacity to enumerate oocysts using only the monolayer resuspension technique enabled the incorporation of ColorSeed into the assay to generate recovery rates and permit the calculation of oocyst densities.

The infectivity of the control oocysts used in this study was slightly higher than previously reported using a similar assay format (14, 15) that did not employ the novel oocyst recovery technique or batch controls. A likely cause for the discrepancy is that the excystation pretreatment batch controls accounted for oocyst losses before inoculation onto the cell monolayer, whereas the previous assay did not. The new assay therefore provides a more accurate measurement of oocyst infectivity.

Recovery rates for our assay across a diverse range of source and wastewater matrices examined were within the typical range (20 to 50%) reported by others (24). Analysis of environmental waters or wastewaters can be challenging; however, only four surface water samples (all replicates of the same composite sample) and one wastewater returned unacceptable recovery results, emphasizing the robustness of this extended assay over a wide variety of matrices. Improved recovery rates for less challenging water matrices, such as filtered waters or waters low in natural organic matter (NOM), may be possible by the removal of the secondary wash step, which is a source of oocyst losses. However, the second-

ary wash is critical for water matrices high in NOM and other contaminants. The initial assay development found that NOM was released from the IMS beads after the oocyst acid dissociation step (data not shown), impacting the availability of essential metal ions in the culture medium, resulting in detachment and loss of the monolayer. Incorporation of a second wash using infection medium removed the majority of these compounds, resulting in successful retention of the cell monolayer. Omission of this step would need to be trialed for any water matrix to ensure that interfering substances are not present.

Application of the integrated assay to rain event samples from the Adelaide Hills agricultural/rural catchment demonstrates its utility for providing more-informative data for use in risk assessments. While oocyst densities were high, in particular for watershed 2 (generally >100 oocysts/10 liters), the overall infectious fraction was only 0.4%. Confidence that this is representative of oocyst infectivity for the samples analyzed is reasonable, as inoculum levels applied to culture were high (overall, >2,000 oocysts). The low level of infectivity was not entirely surprising, given that oocysts can be readily inactivated by a number of environmental stresses (8). None of the 11 infectious foci detected were *C. parvum* or *C. hominis*, with the infectious species detected being associated with either livestock or wild animals, commensurate with land use within the catchment. The results show that the infectivity assay was able to support the growth of a variety of species of *Cryptosporidium*, including *C. ryanae*, *C. ubiquitum*, *C. cuniculus*, and the Rat genotype. While watershed 2 had the highest oocyst densities, the majority of oocysts detected were within the size range of *C. ryanae* (3.16 by 3.73 μm), and both infectious and noninfectious *C. ryanae* isolates were detected at this location, confirming the identification of the small oocysts. *Cryptosporidium ryanae* represents a limited risk to human health, with no recorded human infections. If small oocysts (*C. ryanae*) were included within total oocyst counts, then risk assessments might overestimate the hazard posed to human health.

The incorporation of a simple lysis protocol allows for the PCR-based identification of any infectious *Cryptosporidium* life cycle stages present in the cell monolayer. Based on plate recovery controls, between 10 and 25% of oocysts are not recovered and may remain on the monolayer. Although the extraction protocol is unlikely to efficiently release DNA from intact oocysts, the processing conditions may cause noninfectious oocysts to excyst or be more readily ruptured than an untreated oocyst. To examine this further, we analyzed samples that did not produce any infectious foci, with between 12 and 114 oocysts inoculated onto culture. All of these samples also had ColorSeed oocysts present (4 to 32 oocysts inoculated). Based on the plate recovery efficiencies, these samples had 1 to 29 environmental oocysts and 1 to 7 ColorSeed oocysts remaining on the monolayer. No ColorSeed (*C. parvum*) oocysts were detected by either 18S rRNA or *gp60* nested PCR. Environmental *Cryptosporidium* oocysts were detected by 18S rRNA nested PCR for samples with >11 oocysts remaining on the monolayer, but not for samples with <4 oocysts. This demonstrates the feasibility of obtaining genotype information even if no infectious foci are present, provided that the initial inoculum is high, and also demonstrates that ColorSeed is not a likely source of false-positive signals. These results also suggest that it is possible that oocysts remaining on the monolayer could be a source of interference when typing foci, particularly for samples with 1 small focus or a few foci (with smaller foci having only 16 to 40 life cycle stages present per

focus) and more than 10 oocysts remaining on the monolayer. However, there was no evidence of mixtures present in any of the source water samples typed in this study.

In the case of the melting curve analysis used in this study, it is possible for false negatives to occur when there is overlap in the melting profiles of different species (as is the case for the *gp60* assay) and the signal from a particular species is too low to be detected above the signal of a different species. Such may be the case for plant A anaerobic pot 1, where both *C. hominis* and *C. parvum* were detected by the *lib13* TaqMan assays but only *C. hominis* was detected in the DNA melting curve analysis of the *gp60* amplicon. Similarly, melting curve analysis may show the presence of multiple *gp60* amplicons. These mixes produce ambiguous melting profiles that are difficult to interpret, thus requiring further analysis such as DNA sequencing (or cloning of amplicons and sequencing) to identify the subtypes present.

The integrated assay was successfully applied to a range of wastewater, storm water, and aquifer recharge water samples. Analysis of wastewater samples, particularly raw sewage, evaluated the performance of the integrated assay for analyzing poorer-quality waters. The ColorSeed recovery rates and plate controls for these samples were comparable to those of the rain event catchment water samples. However, unlike oocysts isolated from the catchment water samples, a high proportion of oocysts in primary and secondary effluents were infectious, with the majority of infectious foci identified as *C. hominis* and a much smaller proportion representing *C. parvum*. This reinforces the high human health risk posed by wastewater effluent that has not received appropriate treatment to inactivate oocysts prior to disposal or reuse.

The work described here emphasizes the varying nature of *Cryptosporidium* risk extant in source and wastewaters. This assay should enable comprehensive *Cryptosporidium* risk assessment across source waters and wastewaters, assisting in the direction of appropriate capital expenditure for risk mitigation. While the process is not presented here, this assay also lends itself to the incorporation of a presumptive *Giardia* count if desired. The cost of this assay compares favorably with standard microscopy techniques such as those described in EPA Method 1622. The front end processing costs are the same, and the cost for determining the oocyst count is also similar. The cell culture component represents an additional cost that is prohibitive for the analysis of a single sample but competitive when samples are analyzed in batches of 6 or more, adding approximately 25% additional cost (or less as the batch size increases) to the standard microscopy method. The plate-based format of the cell culture assay allows easier sample handling and processing than do other infectivity assays using Labtek slides. The cost of PCR analysis is also additional and will vary depending on the type of PCR assay used and the level of identification required. From a utility perspective and health regulator perspective, the modest additional cost of the assay is more than offset by the additional infectivity information obtained.

ACKNOWLEDGMENTS

This work was funded by the South Australian Water Corporation, Water Research Australia, and the Victorian Smart Water Fund.

Technical expertise was provided by Melody Lau, Suzanne Hayes, Mira Maric, Ben Chong, and Chris Spry.

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