An Integrated Cryptosporidium Assay to Determine Oocyst Density, Infectivity and Genotype for the Risk Assessment of Source and Re-Use Water

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Abstract

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 measuring oocyst density, infectivity and genotype from a single sample concentrate, an important advance which 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 immuno-magnetic 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 stormwater, 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 waters 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.
INTRODUCTION

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 is 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., USEPA 1622 and 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 re-use, using genotyping assays and, to a lesser extent, infectivity assays (9-13). The information from these assays provide an indication of the risk to of 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, where 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 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 of 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 percent of infectious oocysts, but to also incorporate recovery controls to account for oocyst losses at different process steps. Furthermore, we developed an extraction step to genotype Cryptosporidium lifecycle 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 single format assay for measuring oocyst densities, infectivity and 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 stormwater collection schemes. Here we present results from these activities demonstrating that this single format assay is capable of determining oocyst densities, infectivity and genotype from single grab samples across a variety of waters matrices, thereby providing a more comprehensive understanding of Cryptosporidium risk.

**MATERIALS AND METHODS**

**Single Format Assay Overview**

Figure 1 presents an overview of the work flow for the single format 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 sample, followed by secondary concentration and purification of oocysts by Immuno-Magnetic 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 (Iowa strain) 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 µm pore size) and enumeration using fluorescence microscopy (described in detail...
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-labelled 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 sub-culturing (3 times per week) in RPMI 1640 growth medium with L-glutamine and supplemented with 15 mM HEPES buffer, 100,000 U/L penicillin G, 0.1 g/L 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 (Nunclon™ ∆ Surface) at approximately 4×10⁵ 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 sub-sampled (250 mL – 1000 mL) and diluted in either 5 L or 10 L of reverse osmosis water before primary concentration. Either 5 L or 10 L volumes were processed for source waters or treated wastewaters. Samples (either diluted or neat) were seeded with 100 ColorSeed oocysts immediately prior to concentration, following the manufacturer’s recommendation for adding
ColorSeed to samples. ColorSeed is an internal recovery control, allowing 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 (Dynal, Mulgrave, Australia) following 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 PBS before oocysts were dissociated from the beads by incubating in 50 µL 0.1 N HCl for 10 min at room temperature. The supernatant (containing dissociated oocysts) was then transferred to tubes containing 5 µL 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 µL of acidified water trypsin solution (acidified water (pH 2.4), trypsin (0.025% [wt/vol])) 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 1800 rcf before carefully aspirating 900 µL of the supernatant. The oocyst pellet was washed by two rounds of re-suspension in 900 µL of infection medium, centrifugation for 10 min at 1800 rcf and careful aspiration 900 µL of infection medium. The oocyst pellet was then re-suspended in 900 µL of fresh infection medium pre-warmed to 37°C and inoculated onto cell monolayers that have had the growth medium previously removed. Infection medium consisted of RPMI 1640 medium with L-glutamine, 15 mM HEPES buffer, sodium bicarbonate (2 g/L), glucose (1.0 g/L), bovine bile (0.2 g/L), folic acid (250 µg/L), 4-aminobenzoic acid (1 mg/L), calcium pantothenate (50 µg/L), ascorbic acid (8,750 µg/L), penicillin G (100,000 U/L), streptomycin (100 mg/L), lincomycin (40 mg/L) and gentamicin (50 mg/L). All cell culture reagents were purchased from Sigma-Aldrich, Sydney, Australia. The infection medium was at a pH of 7.4 ± 0.1.

Focus detection method

Oocysts were applied to the cell culture monolayers after excystation pre-treatment and centrifuged onto the monolayer at 408 rcf for 5 min using an Eppendorf (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 pre-warmed infection medium (37°C) and returned to the incubator for a further 44 h. Cell monolayers were then fixed with methanol and stained with SporoGlo (a sporozoite polyclonal antibody which binds strongly to merozoites and all other intracellular reproductive stages [Waterborne Inc, New Orleans, 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 µL of the infection medium within the cell culture well was vigorously but carefully re-pipetted up and down (5-10 times), expelling the medium with each action against the side well 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 re-suspension back into the medium. The infection medium (700 µL) was then removed from each well into separate clean tubes. The remaining infection medium (300 µL) was then removed and combined with the 700 µL volume already recovered. Monolayers were replenished with 1mL of fresh infection medium (pre-warmed to 37°C) and returned to the incubator as described in the above section. The medium collected from each individual cell monolayer was then stained and enumerated as described below.

Filtration counting of oocysts

One mL oocyst suspensions were stained with 30 µL EasyStain (a 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 µm (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 µL of mounting medium (BTF Sydney, Australia) and sealed with a cover-slip. Entire membranes were scanned and all oocysts (ColorSeed and environmental)
were counted by fluorescence microscopy using either Olympus (Notting Hill, 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 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 µL of 0.25% Trypsin-EDTA solution (Sigma [T4049], Sydney, Australia) to each fixed cell culture well and incubating at 37°C for 10 min. Using non-filter tips, the sticky pellet was carefully removed and transferred to a 1.5 mL microcentrifuge tube and centrifuged for 10 min at 20,000 rcf. The supernatant was removed and the pellet washed with 200 µL of 1 × PCR buffer II (Applied Biosystems, Life Technologies, Mulgrave, Victoria) and centrifuged for 10 min at 20,000 rcf. The supernatant was removed and 50 µL 1 × PCR buffer II 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. PCR reaction 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 templates for PCR analysis. Reaction volumes were 25 µL. For nested PCRs, first round reactions 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 000021356 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, UK) care of Dr. Kirsten Elwin and from Murdoch University (Perth, WA, Australia) care of Dr. Una Ryan. DNA melt curve analysis, sequencing and phylogenetic analysis for species identification were performed as previously described (17).

**Excystation pre-treatment and monolayer recovery controls**

To determine oocyst losses during the excystation pre-treatment and to calculate oocyst recoveries from cell monolayers, aliquots of 100 *C. parvum* cattle isolate (Iowa strain) oocysts in 100 µL of PBS were prepared and are referred to herein 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 pre-treatment 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 estimation of oocyst losses on the monolayer (required to calculate the number of oocysts applied to the monolayer). For each experimental batch, 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 to August 2013, of which 8 events were sampled and analyzed. For Watersheds 2, 3, 5 and 7, 10 x 10 L samples were collected from each sampling point for each rainfall event. These samples were then mixed to provide a 100 L composite sample, with quadruplicate 10 L 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 undertaken 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 single format assay as part of a larger project investigating the fate of Cryptosporidium through the wastewater treatment train.

Plant A processes around 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 between January 2013 through 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). Samples (20 x 10 L) were collected on the 28th 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 stormwater samples
The stormwater 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 non-urbanized reserve and rural areas in the Adelaide Hills. This reuse scheme has been designed to harvest up to 350 ML of stormwater per year. Site B is located approximately 15 km north-northwest of Adelaide and collects stormwater from a catchment with industrial and residential inputs. Single 10 L samples were collected from four rainfall-runoff events (>5 mm rainfall) from each site from May 2013 through to 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 oocysts per 10 L). The formula to convert the observed count to a presumptive count per 10 L is described below:

\[
\text{Oocyst Density} / 10 \text{ L} = \left( \frac{\text{Enumerated Oocysts}}{R} \right) \times S \times P
\]

where:

\[
R = \text{Recovery Fraction} = \frac{\text{Colorseed Enumerated} \times P}{\text{Colorseed Added}}
\]

\[
S = \text{Sample volume multiplier} = \left( \frac{10 \text{ L}}{\text{Sample Volume Processed (L)}} \right)
\]

\[
P = \text{Pellet volume multiplier} = \left( \frac{\text{Pellet Size (g)}}{\text{Pellet Processed (g)}} \right)
\]
Calculation of the infectious oocyst fraction

The formula described below was used to calculate the infectious oocyst fraction from a single sample or an entire event:

\[
\text{Infectious Oocyst Fraction (\%) = \left( \frac{\text{Foci Enumerated}}{\text{Oocysts Inoculated onto Culture}} \right) \times 100}
\]

where:

\[
\text{Oocysts Inoculated onto Culture} = \left( \frac{\text{Enumerated Oocysts}}{\text{Plate Recovery}} \right)
\]

\[
\text{Plate Recovery} = \left( \frac{\text{Mean of Plate Recovery Controls}}{\text{Mean of Pre-excystation Controls}} \right)
\]

RESULTS

Oocyst recovery and infectivity for excystation pre-treatment and monolayer batch controls.

Batch controls were developed to determine i) oocyst losses during excystation pre-treatment 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, cells and control live oocysts. The % 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% ± 9 (standard deviation) was achieved for the excystation pre-treatment step, while a mean oocyst plate recovery of 90% ± 12 was achieved for those oocysts inoculated onto plates. Batch 3 was the only batch where 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 only present in 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% ± 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, was incorporated into the single format 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 oocysts densities. Table 2 presents the ColorSeed recoveries of 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% ± 7 was achieved for these samples. Of the 136 individual 10 L samples processed in this catchment study, only four 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% ± 14 for these effluent sources. Out of 84 samples, only 1 returned an unacceptable recovery (<10%). Furthermore, water samples representing an influent aquifer recharge site (Supplementary Table 1) and inlets to two stormwater collection schemes (Supplementary Table 2) were also analyzed using the single format assay, with average recoveries of 37% ± 9 (n=20) and 41% ± 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 densities, with 6 out of 8 events exceeding 100 oocysts/10 L, including Event 2 with numbers exceeding 1000 oocysts/10 L. In comparison the other Watersheds generally had less than 100 oocysts/10 L, with half of the events from Watershed 7 having less than 10 oocysts/10 L.

Interestingly, most of the Cryptosporidium oocysts detected in Watershed 2 stained less brightly with the Easy Stain antibody and were approximately 3.5µm in size, which is outside of the typical size range of 4 – 6 µm for the majority of intestinal Cryptosporidium species.
Some small oocysts were also noted at other sites, but the majority of the Cryptosporidium oocysts detected in Watersheds 3, 4, 5 and 7 were approximately 4.5 µm in size. In total, 2590 oocysts were enumerated from the 136 samples processed. Using the batch controls incorporated into the assay, 2886 oocysts were calculated to have been inoculated onto cell culture. 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 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). Six of the 11 foci detected were determined to be C. ubiquitum, 3 were C. ryanæ and the remaining ones were C. cuniculus and Rat genotype. C. ryanæ, which has a mean size of 3.2 µm x 3.7µm, 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 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. ryanæ. 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 (Figure 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 (approx. $10^5$ oocysts / 10 L) 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 (Figure 3). Based on the density data, oocyst removals across the treatment processes for Plant A, Plant B and Plant C were estimated to be $2.94 \text{ log}_{10}$, 1.12 $\text{log}_{10}$, 1.95 $\text{log}_{10}$ respectively.

Mean oocyst infectivity in the raw sewage for Plants A, B and C was $35\% \pm 0.6$, $52\% \pm 7$, 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, $1.5 \text{ log}_{10}$ oocyst inactivation occurred by the end of Lagoon 2, with at least a further $2 \text{ log}_{10}$ 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 $4.44 \text{ log}_{10}$ (Raw sewage through to Lagoon 4).
For Plant B, secondary treatment did not reduce oocyst infectivity compared with raw sewage. The secondary effluent had approximately 3,000 oocyst/10 L, 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 (Supplementary Table 3) 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 stormwater collection schemes

Of the 20 grab samples collected at an aquifer recharge site, only 1 sample was positive for a single non-infectious environmental oocyst (Supplementary Table 1). In contrast, oocysts were detected in the majority of stormwater samples, with totals of 183 and 156 oocysts and...
mean oocyst densities of 46 ± 26 oocysts / 10 L and 39 ± 42 oocysts / 10 L detected at Site A
and Site B respectively (Supplementary Table 2). 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 1st 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 was most similar to Cryptosporidium sequence 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. (2010, 2012), 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 re-suspension 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 pre-treatment and re-suspension from the monolayers. The latter control was critical for estimating the number of oocysts applied to the monolayer (required for calculating % 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 concentrate 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 labelled 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 ColorSeed (Texas Red labelled) oocysts into such an assay. Our capacity to enumerate oocysts using only the monolayer re-suspension 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 pre-treatment 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 on oocyst infectivity.
Recovery rates for our assay across a diverse range of source and wastewaters matrices examined were within the typical range (20 – 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, may be possible by the removal of the secondary wash step, which is a source of oocyst losses. However, the secondary wash is critical for water matrices high in natural organic matter (NOM) and other contaminants.

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 single format assay to rain event samples from the Adelaide Hills agricultural / rural catchment demonstrate its utility for providing more informative data for use in risk assessments. Whilst oocyst densities were high, in particular for Watershed 2 (generally >100 oocysts / 10 L), 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 >2000 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 x 3.73 µm) and both infectious and non-infectious C. ryanae 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 may 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 – 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 non-infectious oocysts to excyst or be more readily ruptured compared with an untreated oocyst. To examine this further, we analyzed samples that did not produce any infectious foci, with between 12 – 114 oocysts inoculated onto culture. All of these samples also had ColorSeed oocysts present (4 – 32 oocysts inoculated). Based on the plate recovery efficiencies, these samples had between 1 – 29 environmental oocysts and 1 – 7 ColorSeed oocysts remaining on the monolayer. No ColorSeed (C. parvum) were detected by either 18S rRNA or gp60 nested PCR. Environmental Cryptosporidium 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 signal. 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 or a few foci (with smaller foci having only 16 – 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 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 sub-types present.

The single format assay was successfully applied to a range of wastewater, stormwater and aquifer recharge water samples. Analysis of wastewater samples, particularly raw sewage, evaluated the performance of the single format assay for analyzing poorer quality waters. The ColorSeed recovery rates and plate controls for these samples were comparable with 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 was infectious, with the majority of infectious foci identified as C. hominis and a much smaller proportion...
representing *C. parvum*. This re-enforces the high human health risk posed by wastewater effluent that has not received appropriate treatment to inactivate oocysts prior to disposal or re-use.

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 not presented herein, 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 USEPA 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 cost prohibitive for the analysis of a single sample but cost 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 compared with 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 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.
Acknowledgements

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Figure Legends

Fig. 1 An overview of the work flow processes of the Single Format 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 (immune magnetic separation) beads in order to determine densities, infectivity and genotype from a single sample.

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 3-8 (Δ). Watershed 3 Event 3 returned an invalid result due to low ColorSeed recoveries (<10%).

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 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.


17. Webber MA, Sari I, Hoefel D, Monis PT, King BJ. 2013. PCR Slippage Across the ML-2 Microsatellite of the Cryptosporidium MIC1 Locus Enables Development of a PCR Assay Capable of Distinguishing the Zoonotic Cryptosporidium parvum From


22. USEPA. 2010. Quantitative Microbial Risk Assessment to Estimate Illness in Freshwater Impacted by Agricultural Animal Sources of Fecal Contamination. Water UEPAOo,


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Table 1 - Oocyst batch control recoveries and infectivity’s for the single format assay from the Adelaide Hills agricultural / rural catchment

<table>
<thead>
<tr>
<th>Experimental Set-up</th>
<th>Oocysts Recovered after Pre-excystation (%)</th>
<th>Oocysts Recovered from Monolayers (%)</th>
<th>Plate Recovery (%)</th>
<th>Foci Detected (%)</th>
<th>Infectious Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>106 ± 8</td>
<td>93 ± 8</td>
<td>88</td>
<td>53 ± 2</td>
<td>50</td>
</tr>
<tr>
<td>Batch 2</td>
<td>87 ± 3</td>
<td>79 ± 8</td>
<td>90</td>
<td>47 ± 8</td>
<td>54</td>
</tr>
<tr>
<td>Batch 3</td>
<td>90 ± 5</td>
<td>110 ± 11.5</td>
<td>122</td>
<td>61 ± 9</td>
<td>68</td>
</tr>
<tr>
<td>Batch 4</td>
<td>75 ± 19</td>
<td>60 ± 12</td>
<td>80</td>
<td>43 ± 8</td>
<td>57</td>
</tr>
<tr>
<td>Batch 5</td>
<td>89 ± 12</td>
<td>78 ± 6.5</td>
<td>88</td>
<td>38 ± 7</td>
<td>43</td>
</tr>
<tr>
<td>Batch 6</td>
<td>100 ± 13</td>
<td>75 ± 4</td>
<td>75</td>
<td>33 ± 2</td>
<td>33</td>
</tr>
<tr>
<td>Batch 7</td>
<td>94 ± 4</td>
<td>86 ± 4</td>
<td>91</td>
<td>42 ± 6</td>
<td>45</td>
</tr>
<tr>
<td>Batch 8</td>
<td>76 ± 3</td>
<td>65 ± 16</td>
<td>86</td>
<td>31 ± 13.5</td>
<td>40</td>
</tr>
<tr>
<td>Batch 9</td>
<td>92 ± 11</td>
<td>78 ± 8</td>
<td>85</td>
<td>33 ± 7</td>
<td>36</td>
</tr>
<tr>
<td>Batch 10</td>
<td>90 ± 2</td>
<td>80 ± 2</td>
<td>89</td>
<td>39 ± 4</td>
<td>43</td>
</tr>
<tr>
<td>Batch 11</td>
<td>80 ± 6</td>
<td>75 ± 6</td>
<td>94</td>
<td>35 ± 7</td>
<td>44</td>
</tr>
<tr>
<td>Mean</td>
<td>89 ± 9</td>
<td>90 ± 12</td>
<td>47 ± 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The standard deviations of the means are from 3 Batch controls.
Table 2- ColorSeed recoveries (%) for samples analysed from the Adelaide Hills agricultural/rural catchment

<table>
<thead>
<tr>
<th>Rain Event</th>
<th>Watershed 2</th>
<th>Watershed 3</th>
<th>Watershed 4</th>
<th>Watershed 5</th>
<th>Watershed 7</th>
<th>Average Rain Event Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 ± 14</td>
<td>44 ± 7</td>
<td>32 ± 14</td>
<td>37 ± 4</td>
<td>35 ± 5</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>40 ± 20</td>
<td>45 ± 10</td>
<td>60 ± 25</td>
<td>44 ± 14</td>
<td>45 ± 16</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>27 ± 8</td>
<td>1 ± 1*</td>
<td>Δ</td>
<td>43 ± 5</td>
<td>35 ± 13</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>37 ± 7</td>
<td>36 ± 6</td>
<td>Δ</td>
<td>42 ± 7</td>
<td>30 ± 5</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>31 ± 6</td>
<td>36 ± 9</td>
<td>Δ</td>
<td>35 ± 2</td>
<td>23 ± 11</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>33 ± 7</td>
<td>40 ± 11</td>
<td>Δ</td>
<td>37 ± 4</td>
<td>31 ± 9</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>7</td>
<td>36 ± 7</td>
<td>52 ± 5</td>
<td>Δ</td>
<td>46 ± 3</td>
<td>37 ± 7</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>8</td>
<td>29 ± 10</td>
<td>43 ± 7</td>
<td>Δ</td>
<td>38 ± 8</td>
<td>30 ± 5</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>Average</td>
<td>35 ± 6</td>
<td>42 ± 6</td>
<td>46 ± 20</td>
<td>40 ± 4</td>
<td>33 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

Watershed Recovery

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

Δ No samples were collected for Watershed 4 for Rain Events 3-8

*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 analysed from various sampling points along a wastewater treatment plant (WWTP A)

<table>
<thead>
<tr>
<th>Sampling Point</th>
<th>Round 1</th>
<th>Round 2</th>
<th>Round 3</th>
<th>Round 4</th>
<th>Round 5</th>
<th>Round 6</th>
<th>Average Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Sewage</td>
<td>37 ± 2</td>
<td>19*</td>
<td>57 ± 19</td>
<td>49 ± 1</td>
<td>64 ± 8</td>
<td>43 ± 2</td>
<td>45 ± 16</td>
</tr>
<tr>
<td>Anaerobic Pot 1</td>
<td>32 ± 6</td>
<td>20 ± 7</td>
<td>35 ± 4</td>
<td>46 ± 7</td>
<td>60 ± 8</td>
<td>53 ± 11</td>
<td>41 ± 15</td>
</tr>
<tr>
<td>Anaerobic Pot 2</td>
<td>62 ± 6</td>
<td>38 ± 6</td>
<td>39 ± 1</td>
<td>55 ± 6</td>
<td>63 ± 1</td>
<td>54 ± 6</td>
<td>52 ± 11</td>
</tr>
<tr>
<td>Clarifier</td>
<td>39 ± 4</td>
<td>43 ± 5</td>
<td>37 ± 1</td>
<td>46 ± 22</td>
<td>55 ± 12</td>
<td>24 ± 0</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>Lagoon 2</td>
<td>48 ± 0</td>
<td>21 ± 16</td>
<td>31 ± 18</td>
<td>29 ± 3</td>
<td>54 ± 12</td>
<td>17 ± 1</td>
<td>33 ± 15</td>
</tr>
<tr>
<td>Lagoon 4</td>
<td>54 ± 23</td>
<td>33 ± 3</td>
<td>33 ± 4</td>
<td>28 ± 9</td>
<td>34 ± 20</td>
<td>33 ± 13</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>Lagoon 6</td>
<td>40 ± 0</td>
<td>25 ± 6</td>
<td>35 ± 10</td>
<td>26 ± 7</td>
<td>32 ± 1</td>
<td>31 ± 17</td>
<td>32 ± 6</td>
</tr>
</tbody>
</table>

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

*Recovery rate from an individual grab sample as the duplicate was an invalid result.
Table 4 - Characterisation of infective foci detected from the Adelaide Hills agricultural / rural catchment

<table>
<thead>
<tr>
<th>Rain Event</th>
<th>Watershed</th>
<th>18S amplification</th>
<th>GP60 locus amplification</th>
<th>Designation</th>
<th>Phylogenetic Placement</th>
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<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ryanae</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ryanae</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ryanae</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ubiquitum</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ubiquitum</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Positive</td>
<td>C. cuniculus</td>
<td>C. cuniculus</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ubiquitum</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ubiquitum</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>C. ubiquitum</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>Positive</td>
<td>Negative</td>
<td>n.a</td>
<td>Rat genotype</td>
</tr>
</tbody>
</table>

n.a. not applicable
Table 5 – Characterisation of non-infectious oocysts from the Adelaide Hills agricultural / rural catchment

<table>
<thead>
<tr>
<th>Rain Event</th>
<th>Watershed</th>
<th>Oocysts inoculated onto culture</th>
<th>Plate Recovery (%)</th>
<th>18S PCR</th>
<th>gp60 PCR</th>
<th>Species</th>
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<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>104</td>
<td>75</td>
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<td>Negative</td>
<td>C. ryanae</td>
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<tr>
<td>3</td>
<td>2</td>
<td>67</td>
<td>75</td>
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<td>Negative</td>
<td>C. ryanae</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>114</td>
<td>75</td>
<td>Positive</td>
<td>Negative</td>
<td>C. bovis</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>96</td>
<td>75</td>
<td>Positive</td>
<td>Negative</td>
<td>C. bovis</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>44</td>
<td>75</td>
<td>Positive</td>
<td>Negative</td>
<td>C. bovis</td>
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<tr>
<td>1</td>
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<td>Negative</td>
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<td>Negative</td>
<td>Negative</td>
<td>n.a</td>
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<tr>
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<td>19</td>
<td>90</td>
<td>Negative</td>
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<td>n.a</td>
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<tr>
<td>1</td>
<td>2</td>
<td>24</td>
<td>90</td>
<td>Negative</td>
<td>Negative</td>
<td>n.a</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>12</td>
<td>90</td>
<td>Negative</td>
<td>Negative</td>
<td>n.a</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>21</td>
<td>90</td>
<td>Negative</td>
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<td>n.a</td>
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</table>