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Evaluation of an internal positive control for *Cryptosporidium* and *Giardia* testing in water samples

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ABSTRACT

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Aims: An internal positive control for *Cryptosporidium* and *Giardia* monitoring was evaluated for use in routine water monitoring quality control. The control, known as ColorSeed C&G (BTF Pty Ltd, Sydney, Australia), is a suspension containing exactly 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts that have been modified by attachment of Texas Red to the cell wall, allowing them to be differentiated from unmodified oocysts and cysts. The control enables recovery efficiencies to be determined for every water sample analysed.

Methods and Results: A total of 494 water samples were seeded with ColorSeed C&G and with unlabelled *Cryptosporidium* and *Giardia* and then analysed. Additionally, the robustness of the ColorSeed labelling was challenged with various chemical treatments. Recoveries were significantly lower for the ColorSeed Texas Red labelled *Cryptosporidium* and *Giardia* than recoveries of unlabelled *Cryptosporidium* and *Giardia*. However, the differences in recoveries were small. On average ColorSeed *Cryptosporidium* recoveries were 3·3% lower than unlabelled *Cryptosporidium,* and ColorSeed *Giardia* recoveries were 4% lower than unlabelled *Giardia*.

Conclusions: ColorSeed C&G is suitable for use as an internal positive control for routine monitoring of both treated and raw water samples.

Significance and Impact of the Study: The small differences in recoveries are unlikely to limit the usefulness of ColorSeed C&G as an internal positive control. The ColorSeed labelling was found to be robust after different treatments.

Keywords: *Cryptosporidium*, *Giardia*, internal control, quality control, water testing.

INTRODUCTION

Methods available for the detection of *Giardia* and *Cryptosporidium* in water samples generally consist of three stages: sample concentration and elution; separation of cysts and oocysts from other debris; and staining and identification of the protozoan parasites. The methods are technically challenging and the performance of the test method varies between laboratories, analysts and water samples (Clancy *et al.* 1994; Campbell and Smith 1997; Bukhari *et al.* 1998; Sinclair 2000; DiGiorgio *et al.* 2002; Kuhn *et al.* 2002). The variations originate from minor differences in carrying out the testing procedures, and physicochemical properties of water samples such as pH (Kuhn *et al.* 2002) and turbidity (Bukhari *et al.* 1998).

Accurate estimation of recovery efficiency for a particular water sample requires analysis of a duplicate sample, spiked with a known number of *Cryptosporidium* oocysts and *Giardia* cysts (DiGiorgio *et al.* 2002). However, the preparation of precise numbers of *Cryptosporidium* oocysts and *Giardia* cysts is technically challenging because of inherent inefficiencies of the counting techniques (Frederickson 1995) and the fact that oocysts commonly have an uneven distribution within stock suspensions (Bukhari and Smith 1995; Drozd and Schwartzbrod 1996). When counting the number of oocysts

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within replicates of a stock suspension, coefficients of variation in excess of 10 are often obtained (Reynolds *et al.* 1999). Consequently, when using subsequent aliquots of the stock suspension to seed a test system, it is not possible to determine the exact number of oocysts added and therefore it is difficult to interpret recovery data. These problems have been largely overcome by the use of flow cytometry and cell sorting to dispense precise numbers of cysts and oocysts (Reynolds *et al.* 1999; USEPA, 1999; DiGiorgio *et al.* 2002; Sturbaum *et al.* 2002).

Recently an internal positive control for *Cryptosporidium* and *Giardia* testing has become available. The control, known as ColorSeed C&G (BTF Pty Ltd, Sydney, Australia) is a suspension containing exactly 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts that have been modified by attachment of a Texas Red analogue to the cell wall, allowing them to be differentiated from unmodified oocysts and cysts using fluorescence microscopy. ColorSeed C&G is added to a water sample and the sample is analysed as normal. During the final microscopic detection stage of the test, the analyst scans the purified sample for fluorescein isothiocyanate (FITC)-stained oocysts and cysts. Upon sighting an oocyst or cyst in the sample, the filter block of the microscope is changed, and the organism is viewed under red-fluorescence conditions. If the organism is fluorescent red it is counted as a control organism, and if not, the organism is counted as intrinsic to the sample. This process enables the measurement of the recovery efficiency of the test.

The purpose of this study was to evaluate ColorSeed C&G for routine use in monitoring raw and treated water supplies, and determine if recoveries of ColorSeed oocysts and cysts were similar to unlabelled oocysts and cysts. Water samples were seeded with ColorSeed and with a commercially available flow cytometry prepared control known as EasySeed, containing 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts. The EasySeed controls are not fluorescently labelled.

MATERIALS AND METHODS

Sample preparation

Water samples were collected from finished and raw water sampling sites into disposable 10-l polyethylene bladders (Entapack, Dandenong, Australia) and transported to the laboratory. The bladders were prepared with 1 ml of 26% (w/v) sodium thiosulphate (APS Chemicals, Sydney, Australia), in order to neutralize chlorine in finished water samples. Before being used as seeded samples, a duplicate unseeded volume of sample was examined by the same method described below, to ensure that the water did not already contain *Cryptosporidium* or *Giardia*.

The positive control samples were seeded prior to processing with one vial of EasySeed (BTF Pty Ltd, Sydney, Australia) and one vial of ColorSeed (BTF Pty Ltd) according to the manufacturer's instructions. The samples were then processed using membrane concentration.

Membrane concentration

Water samples were concentrated through a 293 mm diameter flat-bed filter unit (Millipore), containing a 2 μm pore size track etched polycarbonate membrane (Osmonics, Minnetonka, MN, USA) using a peristaltic water pump (Watson Marlow unit 604S, Falmouth, UK). Following concentration, the membrane was transferred to a perspex sheet (Micronics, Sydney, Australia). The membrane was then sprayed with elution buffer [0·05% Tween 80 (ICN Biomedicals Inc., Aurora, OH, USA), 2 mM tetrasodium pyrophosphate (Sigma), pH 8] from a wash bottle. The concentrate was eluted using a rubber squeegee (Micronics), and collected in a 50 ml centrifuge tube (Iwaki, Fukujima, Japan). This elution procedure was repeated two to four times, following which the membrane was removed and discarded, and the perspex sheet and squeegee further rinsed with elution buffer. The final volume of the eluted sample was 30–50 ml, and was made up to 50 ml with elution buffer.

The concentrate was centrifuged at 1620 *g* for 10 min at 4°C. The supernatant was aspirated to leave the pellet remaining in a volume of 5 ml, which was then resuspended by vortexing, transferred to a Leighton tube, and further processed by immunomagnetic separation (IMS).

The flat bed apparatus was cleaned between samples by backflushing with a detergent solution (Tergazyme; Alconox Inc., New York, NY, USA) and water. The perspex screen and the squeegee were scrubbed with Tergazyme and rinsed with deionized water.

Following cleaning of the apparatus, all seeded samples were followed during processing by a negative control, consisting of 10 l of deionized water.

IMS and immunoflurescence staining

IMS was performed using the Dynabeads GC-Combo kit (Dynal, Oslo, Norway) according to the manufacturer's instructions, except that following the acid dissociation step the sample was immediately transferred to a 13-mm polycarbonate membrane filter of 0·8-μm pore size (Nuclepore, Jewksbury, MA, USA) mounted on a vacuum manifold. The membrane was then stained with Easystain (BTF Pty Ltd); containing FITC-labelled antibodies specific for *Cryptosporidium* and *Giardia* (Weir *et al.* 2000), according to the manufacturer's instructions; and with a

0·8 mg l^{-1} solution of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) to stain nucleic acids. The membrane was then transferred to a slide, overlaid with the Easystain mounting medium, and a coverslip applied. The slide-mounted membrane was then examined by microscopy.

Microscopy

Sample slides were examined using a Zeiss Axioskop fluorescence microscope (Jena, Germany). The membrane surface was scanned at 200× power for *Cryptosporidium* oocysts and *Giardia* cysts, using an FITC filter set (Zeiss filter set 09). When a green fluorescing cyst or oocyst was detected, a Texas Red filter set (Zeiss filter set 00) was used to differentiate ColorSeed and EasySeed organisms. Bright red cysts and oocysts were counted as ColorSeed organisms. To ensure correct identification, objects were further examined at 400× power, using both a DAPI filter set (Zeiss filter set 02) to allow visualization of nuclei to assist identification, and an FITC filter set when required.

The identification criteria used for *Cryptosporidium* and *Giardia* were as detailed in USEPA Method 1623.

Data analysis

Data analysis was performed using the demonstration version of Graphpad Prism. Data sets were examined for normality using the Kolmogorov–Smirnov test, and examined for difference using paired sample *t*-tests and Wilcoxon matched pairs tests.

ColorSeed fluorescence robustness evaluation

The robustness of the red fluorescence of ColorSeed *Cryptosporidium* and *Giardia* was examined, and compared with the robustness of the antigen recognized by the *Cryptosporidium*-specific and *Giardia*-specific fluorescent antibodies. ColorSeed *Cryptosporidium* and *Giardia* were subjected to a range of treatments and then stained by immunofluorescence and examined for both green and red fluorescence. Aliquots (100 μl) of distilled water that contained 100 Texas Red labelled *Cryptosporidium* and *Giardia* were added to 9 ml of the following solutions: 0·1% sodium hypochlorite, 18·5% (v/v) formalin, 1% (v/v) Tween 80, 1% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) 3-[(3-choloamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS, a non-reducing zwitterionic detergent), 1% (w/v) caprylyl sulphobetaine, 0·1 mol l^{-1} NaOH, 0·1 mol^{-1} HCl, 50% (v/v) ethanol, 50% (v/v) methanol and 50% acetone. The samples were mixed and then incubated at room temperature for 12 h. Further aliquots of ColorSeed *Cryptosporidium* and *Giardia* were boiled for 5 min.

After treatment, the ColorSeed *Cryptosporidium* and *Giardia* were collected onto a 13 mm polycarbonate membrane, stained with Easystain (BTF Pty Ltd), mounted on microscope slides, and cover slipped (similar to the procedure described above). Slides were then examined by fluorescence microscopy as described above, and the level of green fluorescence and red fluorescence subjectively assessed.

RESULTS

Recovery comparison

A total of 247 raw water and 247 finished water samples were spiked, and examined using the procedure described above, between January and October 2002. The resulting data was compiled into data sets comparing recovery of ColorSeed organisms to EasySeed organisms; for *Cryptosporidium* and *Giardia*; for each of the water types, and in a combined data set of both water types.

The data sets were examined for normality using the Kolmogorov–Smirnov test (Graphpad Prism, demonstration version). The raw water and finished water *Cryptosporidium* data sets both showed normal distribution ($P > 0·05$), while the combined water *Cryptosporidium* data set showed non-normal distribution ($P < 0·05$). At least one of each pair of the *Giardia* data sets showed non-normal distribution ($P < 0·05$).

For paired samples *t*-tests were performed on the data sets showing normal distribution (*Cryptosporidium* raw and finished waters), and Wilcoxon matched pairs tests performed on the other data sets (Graphpad Prism), with the null hypothesis that there was no difference between the data sets. The results are presented in Table 1.

The Easyseed counts for both organisms were significantly higher ($P < 0·01$) than the ColorSeed counts. The actual difference in mean values ranges from 3 to 5%. Variation in counts was similar for EasySeed and ColorSeed, for both organisms, in both water types. Combining the raw and finished water data sets confirms the observations of significantly greater counts for EasySeed than for ColorSeed ($P < 0·01$).

The effects of background material on target organism recovery were relatively consistent between the compared sample sites, as could be expected from good quality source waters and the resultant product waters. The finished waters were predominantly taken from three sample sites, with average EasySeed *Cryptosporidium* recoveries of 60, 67 and 68%; and average EasySeed *Giardia* recoveries of 72, 75 and 76%. The raw waters were predominantly taken from five sample sites, with average EasySeed *Cryptosporidium* recoveries of 41, 49, 60, 62 and 63%; and average EasySeed *Giardia* recoveries of 76, 76, 79, 84 and 90%. At all sites the

Table 1 Comparison of percentage recoveries of EasySeed and ColorSeed seeded into the same water samples

Sample type	ES [mean (S.D.)]	CS [mean (S.D.)]	$P(T<=t)$
Cryptosporidium [raw waters (n = 247)]	61% (22)	58% (19)	0·0042
Giardia [raw waters (n = 247)]	81% (13)	76% (14)	<0·0001
Cryptosporidium [finished waters (n = 247)]	65% (16)	61% (17)	<0·0001
Giardia [finished waters (n = 247)]	75% (14)	72% (15)	<0·0001
Cryptosporidium [all samples (n = 494)]	63% (19)	60% (18)	<0·0001
Giardia [all samples (n = 494)]	78% (14)	74% (15)	<0·0001

ES, EasySeed; CS, ColorSeed; $P(T<=t)$, probability of null hypothesis being supported.

ColorSeed recovery was slightly lower than the EasySeed recovery.

ColorSeed fluorescence robustness evaluation

Examination of ColorSeed *Cryptosporidium* and *Giardia* that had been exposed to a range of treatments showed a level of red fluorescence comparable with untreated samples. Green fluorescence was bright and comparable with the untreated samples for all treatments except for the samples that were treated with 0·1% sodium hypochlorite, 1% SDS and 0·1 mol l^{-1} NaOH; these samples all showed a significantly reduced level of green fluorescence. The results for both cysts and oocysts were similar.

DISCUSSION

The results of this study demonstrate that ColorSeed C&G is suitable for use as an internal positive control for routine monitoring of raw water samples and drinking water samples for *Cryptosporidium* and *Giardia*. A total of 494 water samples were seeded with ColorSeed C&G, unlabelled *Cryptosporidium* and *Giardia*, and then analysed. Recoveries were significantly lower for the ColorSeed Texas Red labelled *Cryptosporidium* and *Giardia* than recoveries of unlabelled *Cryptosporidium* and *Giardia*. However, the differences in recoveries were small. On average for all samples, ColorSeed *Cryptosporidium* recoveries were 3·3% lower than unlabelled *Cryptosporidium,* and ColorSeed *Giardia* recoveries were 4% lower than unlabelled *Giardia*. These small differences in recoveries are unlikely to limit the usefulness of ColorSeed C&G as an internal positive control.

The reason that the Texas Red labelled *Cryptosporidium* and *Giardia* produce slightly lower recoveries is probably because of the increased hydrophobicity of the labelled oocysts resulting in increased attachment to surfaces and losses during the concentration or analysis. Initial studies using microbial adhesion to hydrocarbons (MATH) methods (derived from Drozd and Schwartzbrod 1996) have indicated that the Texas Red label may affect the hydrophobicity of cysts and oocysts (data not shown).

The Texas Red labelling is stable after exposure to a wide range of conditions. In fact, the Texas Red labelling appears to be more robust than the surface antigen recognized by the *Cryptosporidium*-specific and *Giardia*-specific monoclonal antibodies. It has been reported previously that oxidizing conditions can affect the surface antigen recognized by all the available *Cryptosporidium*-specific antibodies (Moore *et al.* 1998) and result in poor staining.

The method used in this study to analyse water samples for *Cryptosporidium* and *Giardia* is a modified form of USEPA Method 1623 that results in improved recoveries. Flat bed membrane filtration (derived from Ongerth and Stibbs 1987) is used to concentrate water samples instead of the PALL-Gelman Envirochek filter capsule described in Method 1623. Following the IMS process the sample is collected onto a 13-mm diameter membrane, and immunofluorescence and DAPI staining is performed on the membrane. This overcomes the losses that occur because of oocysts and cysts washing off the slide (Reynolds *et al.* 1999) and improves immunofluorescence staining (data not shown) but it has the disadvantage that it does not enable the use of differential interference microscopy (DIC) to confirm oocysts and cysts. However, unlike other immunofluorescent staining reagents, the one used in this study uses highly specific IgG1 subclass antibodies (Weir *et al.* 2000) that do not cross react with algal or debris particles (Ferrari *et al.* 1999). When these reagents are used in conjunction with DAPI staining as a confirmation, DIC confirmation is not required. This is in agreement with the conclusion of Smith *et al.* (2002) that DAPI staining assists more in oocyst identification than DIC microscopy.

The use of ColorSeed within large scale, multilaboratory monitoring programmes such as the regulated monitoring of drinking water in the UK and the proposed LT2 programme to monitor surface water in the USA would greatly improve the quality of the data generated. The return of a quality control result from every sample tested enables the performance of the testing to be measured precisely, both within each laboratory, and between laboratories. The use of ColorSeed may not add significant cost to the total programme, and almost certainly saves on the expense associated with managing water supply quality using

inaccurate data. It would seem that the use of ColorSeed in every sample tested can result in unchanged or reduced analytical costs because of the elimination of some quality control samples.

When using methods that differ from the one described here, recovery experiments should be performed to check that similar recovery of ColorSeed, and unlabelled cysts and oocysts are obtained.

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