

Proficiency testing of *Cryptosporidium* and *Giardia* analyses – an Australian case study

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Abstract An inter-laboratory proficiency trial was undertaken as a prelude to a survey of Australian drinking source waters for *Cryptosporidium* and *Giardia* oo/cysts. Specific aims included measuring the recovery of protozoan control material from representative source waters, identifying laboratory errors and assessing the utility of pre-stained reference oo/cysts. Five experienced laboratories were provided with coded vials containing oo/cysts or blanks for spiking 10 L water samples (11 replicate environmental, 5 filtered) or to be analysed directly (16 Count Control vials). Each laboratory reported both their standard counts and details of how each oo/cyst image was interpreted and confirmed. A sixth laboratory provided flow cytometry prepared seed material and reference analyses. Average recoveries for 10 L samples ranged from 0–67% and 0–72%, for environmental and filtered tap water respectively, depending on laboratory and sample type. The best performing laboratory obtained high recoveries, ranging from 45% to 66% for tap water and 63% to 81% for environmental water. Nonetheless, recovery from tap water and environmental sample analysed simultaneously by the same laboratory could differ markedly (1% v. 48% in one case). Poor recovery and false positives were encountered despite the experience of the participants. Inconsistencies in reporting were introduced by the different procedures for confirming oocysts and cysts. The trial showed that obtaining reliable estimates of environmental protozoan loads is still a concern for water managers.

Keywords *Cryptosporidium*; *Giardia*; proficiency; quality control

Introduction

Measurement and management of the protozoan parasites *Cryptosporidium parvum* and *Giardia lamblia* in drinking source waters are major issues for water authorities worldwide. Following the 1998 water supply incident in Sydney, Australia, concerns were raised about the reliability of local laboratory methods (Clancy, 2000). In response, the Australia/New Zealand National Association of Testing Authorities (NATA) in association with the Water Services Association of Australia are coordinating a series of inter-laboratory comparisons to develop performance standards for accrediting laboratories. Disturbingly, initial trials, which utilised a low level of sample replication, highlighted concerns. Even when analysing clean water samples often using procedures recommended in method 1623 (US-EPA, 1999), over 50% of the participating laboratories were substandard in their ability to detect these pathogens (NATA, 1999, 2000a, 2000b). Problems included false positives, false negatives and poor/highly variable detection rates. Similar problems have also been reported in North America (Simmons *et al.*, 1999) and the UK (Bouchier, 1998).

Such difficulties in reliably measuring parasite concentrations, assuring consistent analytical performance, and the resulting difficulty in comparing data from different labs provide motivation to develop and test new QA/QC tools. The work described here was conducted as part of a project to measure pathogen presence in drinking water sources in different parts of Australia. To ascertain the proficiency of the prospective contracting laboratories an initial proficiency trial was designed with the following aims:

- assess the reproducibility of oo/cyst spike recovery from source water samples, representative of those which they would routinely test, and of locally filtered tap water;
- measure the effect of representative source waters on seed recovery;
- identify and measure the extent of any methodology deficiency for each laboratory;
- determine the consistency between count data routinely reported and an analyst's interpretation; and
- assess the robustness and behaviour of 2 spike preparations for quality control of oo/cysts.

Methods

Proficiency trial design

Five contracting laboratories were selected to each analyse 11 replicate samples from a regional drinking water supply after spiking with varying concentrations and combinations of *Cryptosporidium* and *Giardia* oocysts or blanks. Vials of the seed spike materials and blanks termed "Count Controls" were also analysed. At the conclusion of analytical work 4 additional vials of seed material ("Trip Controls") were forwarded to a separate reference laboratory (BTF Pty. Ltd.) for measuring transport and storage impacts. As far as practicable the contents of seed material and the information sought (Table 1) were not revealed to the analysts.

Trip Control data were compared to assess seed quality. Count Controls were used to assess whether laboratories could consistently dispense the seed and count oo/cysts to a standard comparable to the reference laboratory. Filtered water sample data were used to assess whether laboratories could extract oo/cysts from a filtered water (5 × 10 L) and to compare their relative performances on a similar water matrix. Source water (11 × 10 L) sample data were used to evaluate the impact on oo/cysts recovery with other environmental water constituents.

Water samples

Environmental samples were collected during dry weather from selected catchments and aquifers. Each laboratory provided their own sterile filtered water. Resource limitations prevented simultaneous collection of other water quality data. However, a range of water quality parameters were measured at the same sites under comparable dry weather conditions, during the proficiency trials (Table 2).

Seed vial contents and coding

Five protozoan seed vial batches, were specially prepared by BTF for the proficiency trials (Table 3). Four were derivatives of BTF's principal quality control product EasySeed™, which contains 100 oo/cysts of both *Cryptosporidium* and *Giardia* (<http://www.biotech-frontiers.com/>). A fifth set (blanks) contained only distilled water. All vials contained 1–2 mL of filtered deionised water and had been gamma irradiated.

BTF were developing Texas Red labelling of oo/cysts as a possible pre-stain for spiking

Table 1 Information sought from the method proficiency trial

<ul style="list-style-type: none"> • Statistics on seed recovery (e.g. mean, median, range, standard deviation) for different laboratories, sample types, seed types, spike sizes • Quality of the seed material and effect of storage and transport • Features of well performing laboratories 	<ul style="list-style-type: none"> • Evidence of laboratory problems e.g. false positives, false negatives/low recoveries, miscoding/misreading, inconsistent reporting, failure to achieve target performance levels, poor seed inoculation and staining • Effects of using 2 seed preparations on the quality of recovery data • Practicality of survey logistics
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Table 2 Typical dry weather analysis of water examined during parallel proficiency trials

Water source Sample type	Creek 1 Surface	Creek 2 Surface	Creek 3 Surface	Aquifer 1 Ground water	Laboratory Filtered tap water
Predominant land use	Peri-urban/ woodland	Agriculture	Forest	Urban	–
Turbidity (NTU)	15	1.1	2	1	<0.5
pH	7.8	7.8	5.7	5.6	5.5–6.0
Conductivity ($\mu\text{S}/\text{cm}$)	300	470	30	33	< 2
UV absorbance (254 nm 1 cm cell – % transmission)	30	78	71	70	100
True colour (Pt Co)	90	14	40	20	<1
<i>Escherichia coli</i> 100 mL ⁻¹	200	80	700	0	0
Enterococci cfu.100 mL ⁻¹	400	40	200	0	0
<i>Clostridium perfringens</i> cfu.100 mL ⁻¹	90	2	100	80	0

Table 3 Seed vials contents

Seed Vial	Organism	Total count \pm (std deviation)	Mean DAPI confirmed count	Pre-stain*
CX1	<i>Cryptosporidium</i>	149 \pm 0.8	144.5	No
CX2	<i>Cryptosporidium</i>	64 \pm 0.8	61.4	Yes
GX1	<i>Giardia</i>	174 \pm 1.6	174	No
GX2	<i>Giardia</i>	79 \pm 0.8	77.4	Yes
BL	Neither	0	0	–

* Pre-stained with Texas Red

material to distinguish spiked oocysts in the presence of native oo/cysts. They wished to assess the effects of pre-staining oo/cysts and the proficiency trial was seen as an opportunity for characterising the prototype material.

Prior to transport to the testing laboratories, seed vials were divided into sets identified only by a unique alphanumeric code. The first letter of each code set indicated its use as follows: “S” (for environmental sample inoculum), “B” (for filtered water inoculum), “C” for “Count Control” and “T” for Trip Control (Table 4). Sufficient replication (11, 5, 16, 2 respectively for S, B, C and T) was incorporated to allow calculation of descriptive and comparison statistics (e.g. mean, standard deviation, t-statistics).

Inter-laboratory cooperation

The five prospective contracting laboratories, were selected for being experienced and having provided commercial protozoan testing for major Australian water authorities. Each laboratory was instructed to use its own in-house method, to analyse 10 L water samples, and enumerate “Count Controls” using BTF’s quality control method (<http://www.biotech-frontiers.com/>) and equipment. The major differences between laboratory methods were in the concentration step, where Envirochek™ filters, 293mm flat bed filtration or calcium flocculation were used.

A result scoring sheet was supplied with instructions for recording every oo/cyst morphology. Completed sheets provided details on whether each oo/cyst was a presumptive *Cryptosporidium* or *Giardia* oo/cyst; its antibody staining intensity (+, ++ or +++); the presence and number of DAPI stained nuclei; the presence of other “typical” morphology; and the microscopist’s conclusion as to whether the oo/cyst was confirmed or not.

In line with most inter-laboratory comparisons it was agreed not to disclose the identity of the participating laboratories in open literature documents or in reports provided to all

Table 4 Contents of vial sets provided to each organisation

Coding	Set description	Vial set
'S' followed by 5 digit code	4 vials per set, each set to spike a 10 L source water sample (total 11 sets)	1 CX1, 1 CX2, 1 GX2, 1 BL vials (2 sets) 1 GX1, 1 CX2, 1 GX2, 1 BL vials(2 sets) 1 CX1, 1 GX1, 1 GX2 and 1 CX2 vials (3 sets) 1 CX2 and 3 BL vials (2 sets) 1 GX2 and 3 BL vials(2 sets)
'B' followed by 5 digit code	4 vials per set, each set to spike a 10 L purified (deionized) water sample (total 5 sets)	1 CX1, 1 CX2, and 2 BL vials (2 sets) 1 GX1, 1 GX2, and 2 BL vials (2 sets) 1 GX2, 1 CX2, 2 BL vials (1 sets)
'C' followed by 5 digit code	1 vial per Count Control code set containing 1–2 mL of seed (total 16 sets)	1 CX1 vial (3 sets) 1 GX2 vial (3 sets) 1 CX1 vial (3 sets) 1 GX2 vial (3 sets) 1 dummy vial (4 sets)
'T' followed by 5 digit code	1 vial per code set containing 1–2 mL of seed for Trip Controls (total 2 sets)	1 CX1, 1 GX1, 1 GX2 and 1 CX2 vials (2 sets, one for analysis, the other for contingencies)

contractors. The exception to this arrangement was the reference laboratory, BTF, who as providers of the spiking materials, knew of the initial composition of the spike vials. They also analysed all Trip Controls. BTF, however, were not involved in the design of the inoculum sets or coding of spikes and were not aware of the returned contents of the Trip Control vial sets, which included blanks.

Results and discussion

Overall findings

Key statistics are presented in Table 5. For ease of comparison, data are expressed in terms of percent recovery of “total” FITC antibody labelled oo/cysts, and confirmed oo/cysts. Data is also presented summarising the background levels of oo/cysts, and the occurrence of false positives and false negatives. All statistics relate to oo/cysts spiked into waters prior to processing. In only one case did an environmental water sample contain low counts of oocysts (1/19, Table 5). In this instance, *Cryptosporidium* (10 in 10 L) were detected in an environmental sample by Laboratory 3. Percent recovery figures were adjusted for a background count of 5 oocysts per 10 L by subtraction of five from all confirmed counts. The “background” *Cryptosporidium* count reported by Laboratory 5 was not considered reliable as it was comparable in size to false positive counts for blank samples. Laboratory performance ranged from excellent (recovery of 50–60% of seeded oo/cysts from both environmental and filtered water samples, low standard deviation, nuclear morphology distinct, no detectable problems with count or Trip Controls) to poor (poor recoveries, high relative standard deviations for recovery data, nuclear morphology absent, some false positives) (Table 5).

Performance of water analysis laboratories

Seed stability. As seed sets were assembled at the same time, but used over a 6 month period, their stability was assessed by comparing total and confirmed “Trip Controls” counts. This analysis indicated that all spike material was stable over the trial in respect to morphology, nuclear and antibody staining. Total and confirmed counts averaged approximately 90% and 80% of their original values respectively for both *Cryptosporidium* and *Giardia*. Except for a slight post production drop, the numbers of seed cysts and oocysts did not decrease significantly as the spike material aged. BTF use a novel process

Table 5 Selected summary results from amalgamated counts of vials and seeded samples

Organ-ism	Labor-atory	Sample/control type	% Recovery				Oo/cysts per 10 L		
			Mean ± 1 SD (total)	Range (n) (total)	Mean (total)	Mean (conf.) (X2 only*)	Back-ground (conf.)	False -ve (conf.)	False +ve (conf.)
<i>Cryptosporidium</i>	1	Trip	95 ± 1.6	94,96(2)	94	85	-	0	0
		Count	73 ± 25	34-97(5)	79	-	-	-	0*
		Blank	20 ± 26	0-48(3)	0	0.0	-	3	0
	2	Sample	25 ± 19	0-50(9)	26	0.5	0,0	7	-
		Trip	97 ± 2	96,98(2)	98	89	-	0	0
		Count	92 ± 18	89-95(6)	94	-	-	-	0*
	3	Blank	71 ± 9	63-81(3)	81	72	-	0	0
		Sample	55 ± 7	45-66(9)	61	54	0,0	0	-
		Count	76 ± 16	54-96(6)	80	79	-	0	0
	4	Blank	5.8(2,3)	4.2-8.5(3)	5	5.8	-	0	0
		Sample	24 ± 12	0-48(9)	17	24	0,10	1	-
		Trip	84 ± 3	81,86(2)	86	84	-	0	0
	5	Count	87 ± 19	59-119(6)	69	90	-	0	2
		Blank	47 ± 6	42-53(3)	50	49	-	0	0
		Sample	1.1 ± 1.1	0-3.3(9)	0	1.2	0,0	3	-
	6	Trip	78 ± 0.2	78,78(2)	78	61	-	0	0
		Count	53 ± 21	11-67(6)	46	-	-	0	3
		Blank	10 ± 6.4	3-16(3)	8	9	-	0	2
<i>Giardia</i>	1	Sample	6.8 ± 4.4	3-13(9)	3.5	4.2	0,4	1	-
		Trip(all)	89 ± 8	78-98 (10)	91	80	-	0	0
		Count	95 ± 3.7	92,98(2)	92	87	-	0	0
	2	Count	73 ± 43	9-122(6)	57	-	-	-	0*
		Blank	23 ± 20	0-36(3)	0	0.0	-	3	0
		Sample	15 ± 7	0-15(9)	0.0	0.0	0,0	9	-
	3	Trip	97 ± 4.5	94,100(2)	94	89	-	0	0
		Count	98 ± 1	96-100(6)	98	-	-	-	0*
		Blank	54 ± 7	46-59(3)	57	42	-	0	0
	4	Sample	67 ± 9	58-87(9)	63	50	0,0	0	-
		Count	103 ± 10	86-114(6)	88	104	-	0	0
		Blank	5.2 ± 6.7	0,9.9(2)	-	4.7	-	1	0
	5	Sample	25 ± 18	0.4-57(9)	17	25	0,0	0	-
		Trip	87 ± 0.1	87,87(2)	87	66	-	-	0
		Count	90 ± 9	73-99(6)	83	91	-	0	0
	6	Blank	47 ± 0.5	47-48(3)	41	48	-	0	0
		Sample	49 ± 16	28-79(9)	16	50	0,0	0	-
		Trip	85 ± 6	81,89(2)	81	74	-	0	0
7	Count	73 ± 7	63-81(6)	68	-	-	0	2	
	Blank	3.5 ± 3.6	1.2-7.6(3)	5	3.0	-	0	0	
	Sample	1.1 ± 0.6	0-2(9)	0	0.1	0,0	8	-	
8	Trip (all)	91 ± 6	81-100(10)	78	78	-	0	0	

Notes:

- 1) Statistics are calculated with reference to different counts as follows:
 "1 SD" = 1 standard deviation
- 2) "total" refers to total counts i.e. presumptive FITC antibody positive count.
 "conf." = confirmed by DAPI or DIC
- 3) Statistics were calculated on the combined X1 + X2 counts, except where indicated (i.e. "X2 only" column) (* see Table 3 for description of spikes)
- 4) Counts for Trip Controls (T) were all measured by BTF

for stabilising the seeds that includes gamma irradiation. It appears that this process results in a seed preparation that is stable for at least 6 months at 4°C.

Inoculation of seed. For a successful trial it was essential that a high number of both seed types was inoculated into each 10 L water sample. Comparison of Count and Trip Control data showed that the contracting laboratories were able to dispense on average > 73% of the

initial seed vial contents and recognise and reliably count them. Laboratory 1 appeared to have some difficulty in doing this consistently. In the latter case some *Giardia* and *Cryptosporidium* seeds were apparently poorly mobilised (indicated by some low % recoveries).

Recovery of seed from filtered tap water. Counting of oo/cysts in 10 L of purified water provided a comparison of how well different laboratories could recover seeds when there was no interfering matter to obscure oo/cysts. Recovery varied greatly between laboratories ranging from a high of 70% to a low average recovery of $\leq 10\%$.

Recovery of seed from environmental samples. The pattern of recovery obtained with purified water was largely, though not entirely, reproduced with environmental samples. Laboratory 2 recovered on average $> 50\%$ and achieved a tight standard deviation with both protozoa. Laboratory 1 obtained a reasonable recovery of total seed of around 20% but, as with the filtered water, it also reported a low confirmation rate and a high standard deviation. Laboratory 5 again obtained low recoveries of both seed types.

The most marked difference was with Laboratory 4's *Cryptosporidium* counts. Laboratory 4 recovered $< 2\%$ from the environmental water samples and greater than 42% for the sterile reagent water. This effect was observed with all 9 seeded environmental water samples but was not evident with the *Giardia* counts. It is suggested that particulate material, reported by the laboratory as a hindrance to analysis, interfered with the detection of *Cryptosporidium* oocysts in this particular water type. Smaller, though still statistically significant ($p < 0.05$), were the higher *Cryptosporidium* and *Giardia* count differences obtained by Laboratory 3 in environmental water samples than in sterile reagent water. These differences demonstrated firstly the need for frequent use of positive controls (i.e. spiking) when analysing any environmental sample and routinely reporting such data. As a minimum both percent recovery of oo/cysts and the variance of such counts should be reported e.g. as a standard value. This would provide simultaneously a correction factor to estimate total oo/cyst numbers and a measure of laboratory proficiency.

Count confirmation. Confirmation of Count Controls was high for Laboratory 3 and Laboratory 4. Due to some confusion regarding instructions on how to undertake Count Control staining the other laboratories did not fully confirm whether the intracellular contents of the Count Control oo/cysts were present, though BTF's analysis of the analogous Trip Controls indicated that high confirmation should have been expected. In the case of the filtered water, total counts were comparable to confirmed counts except in the case of Laboratory 1 where low confirmation rates were reported. This pattern was repeated with confirmed counts. Discussions with Laboratory 1 indicated that its DAPI staining procedure was not optimised. This raised the question of where confirmed native oo/cyst numbers might have been underestimated.

False negatives. Four of the five laboratories reported false-negative results for *confirmed counts* performed on 10 L water samples. In one instance (Laboratory 4) it was proposed that this was due to constituents of the environmental water sample. In the 3 other instances the likely cause was some aspect of the analysis procedure or its variability as reflected in high standard deviations. The number of false negatives reported by Laboratories 1 and 5 were a concern.

False positives. *Confirmed* false positives were reported by 2 laboratories. Examination of Laboratory 4 data sheets showed that the higher false positive count (5) was due to an off

the filter group of oocysts and this had been flagged by the analyst. The source proposed was contaminated mounting medium. The source of false positive *Cryptosporidium* by Laboratory 5 was unclear. Though low in number (1–10 oocysts/10L) the counts were significant as they were comparable in size to the numbers reported for the environmental samples analysed by Laboratory 5.

Interpretation of microscopy

Examination of records for the 13,500 oo/cysts examined revealed important information typically absent from basic summary reports. Examination of nuclei counts indicated good condition of the seeds with 92% of confirmed *Cryptosporidium* and 96% of *Giardia* having 2 or more nuclei. They also showed that Count Controls were not always confirmed and different interpretations of the meaning of “confirmed” were used. In one instance the confirmation process was insufficiently documented, and discussions with the analyst indicated oversight resulting from operator fatigue.

Behaviour of Texas Red pre-stained material

A variety of data were collected on the Texas Red pre-stained oo/cysts. Trip Control and Count Control data indicated that seed morphology, staining and structural integrity were good and comparable to non-Texas Red material. ANOVA comparisons of percent recovery of seed from environmental samples having different proportions of “X1” and “X2” seed types showed either no or only minor differences (compare “X1 + X2” and “X2 only” counts in Table 5). *Cryptosporidium* but not *Giardia* were selectively obscured in Laboratory 4 environmental samples, irrespective of whether the oocysts were pre-stained or not, indicating that their interaction with particulate material was comparable. This was supported by measurement of seed material isoelectric points and electrophoretic mobility. In distilled water and PBS no significant surface charge differences were detected between material with or without Texas Red pre-staining (unpublished data).

Initial examination of mixed Texas Red positive and negative material showed that the well pre-stained oo/cysts could readily be distinguished and counted from one another in the presence of particulates. Subsequently, some dimming was reported of the Texas Red fluorescence of X2 experimental material. BTF have now refined the production of Texas Red labelled material, and subsequent reports from laboratories in the CRC study indicate the pre-stained material is now easily detected in spiked environmental samples (unpublished data). This material is currently being released as an internal sample control commercially under the name ColorSeed™.

Laboratory feedback

Fatigue. Discussion with laboratories about their experience raised a number of issues. While the use of internal controls has clear advantages, the fatigue and cost involved in distinguishing, counting and confirming up to several hundred oo/cysts in every sample needs to be considered and may raise the cost of each analysis, particularly if individual oo/cyst data were sought.

Achieving agreed performance targets. NATA is currently proposing that laboratories aim for recoveries of between 10 and 110% with no false positive or negative results. While this seems reasonable it does not allow for interference which may arise from particulate and solute material. By the above criterion Laboratory 4 recoveries of *Cryptosporidium* from environmental samples would have been unacceptable even though overall assessment of all data sheets indicated they were competent analysts. Sensitivity of contract laboratories to disclose detailed data sheet information or guarantee a predetermined level of perform-

ance was evident. Even, Laboratory 2 was unwilling to provide a pre-trial performance guarantee for what was a relatively clean environmental water sample.

Conclusions

Laboratory performance in analysing parasitic protozoan oo/cysts is still highly variable across Australia, and probably elsewhere. Consequently, the quality of data generated by a laboratory cannot be taken for granted by water managers who need to become active participants in the quality assurance process. For example managers should not collect pathogen counts even from certified laboratories using standardised methods without also obtaining and examining quality control data and details of the oo/cyst confirmation methods.

This caution applies even when a laboratory can be shown to be performing excellently with standard samples as this is no guarantee that analyses of environmental water will provide a reliable result in the absence of project specific quality control information. We strongly recommend that managers insist on greater use of blanks as well as spikes. Data on spiking should always report % recovery \pm standard deviation or equivalent. One way to obtain QC data would be the widespread use of internal quality controls. BTF's new ColorSeed™ appears to be a suitable internal quality control material. It combines the distinctiveness of a control material with very high similarity to the material of interest. Tests undertaken in this study on prototype material confirm its potential.

Overall the results demonstrated that the routine inclusion in our ongoing survey, of replicates, blanks and spiked samples is essential to assessing water quality under different climatic, geographical and land-use conditions.

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