

TESTING METHODS FOR DETECTION OF *CRYPTOSPORIDIUM* SPP IN WATER SAMPLES

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Abstract. A large waterborne outbreak of cryptosporidiosis in Milwaukee, Wisconsin, USA in 1993 prompted a search for ways to prevent large-scale waterborne outbreaks of protozoan parasitoses. Methods for detecting *Cryptosporidium parvum* play an integral role in strategies that lead to appropriate treatment of surface water, but are criticized because they produce results that are highly variable.

The US Environmental Protection Agency developed a set of criteria to evaluate detection methods for protozoan parasites in water. As a consequence, the Agency has had to develop approaches to reducing uncertainty of evaluations. The variability and accuracy of various methods of producing small numbers of *Cryptosporidium* spp oocysts were tested. The least variable and most accurate method was used to spike seven surface water, and one tap water sample to compare 4 detection methods that had been reported in the literature. The least variable and most accurate method for spiking specified numbers of oocysts into samples was found to be flow cytometry. The most effective of the methods tested for detection in surface, tap and reagent water was solid phase cytometry.

INTRODUCTION

Cryptosporidium parvum is a well-known cause of waterborne disease. This coccidian protozoan parasite may contaminate water from either human or zoonotic sources. This disease reached prominence in the United States after an outbreak in Milwaukee that sickened an estimated 400,000 individuals with 54 immune compromised individuals dying as a result. This outbreak was traced to the consumption of contaminated drinking water (MacKenzie *et al*, 1995). Largely as a result of this outbreak, the United States Environmental Protection Agency (US EPA), received a mandate from Congress to develop appropriate regulations to prevent a recurrence of this type of outbreak. To fulfill this mandate, the US EPA after negotiation with various stakeholders, planned and undertook a large-scale study under the Information Collection Rule (ICR). The ICR was designed in part to determine the occurrence and distribution of *Cryptosporidium* spp in source and drinking waters in the United States (National Primary Drinking Water Regulations, 1996). The methodology used for detection of *Cryptosporidium* spp in this study has been recognized (Allen *et al*, 2000) to produce highly variable results, with low recovery of presumptive oocysts. The method relies on antibody detection that may lead to overestimation of oocyst concentration due to the possibility of cross reactivity of the antibody with unrelated organisms (Rodgers *et al*, 1995). In order to reduce this overestimation, it is necessary to confirm any oocyst like objects detected by immunofluorescence. The method implemented in the US EPA's ICR used differential interference microscopy on samples spotted on membranes cleared

by dehydration and glycerol saturation. This procedure results in confirmation of very few oocyst-like objects as belonging to *Cryptosporidium* spp.

Even while the ICR study was being conducted, widespread recognition of the weaknesses of the method for detection of protozoan cysts and oocysts (Fricker and Crabb, 1998) led to development of alternative methods by a variety of laboratories. These methods have included molecular detection (Sluter *et al*, 1997), cell culture methods (Slifko *et al*, 1999), direct counting (Reynolds *et al*, 1999), and hybrid methods such as cell culture methods paired with molecular detection techniques (Di Giovanni *et al*, 1999). The problem of adopting these methods directly for use in occurrence studies is that they were not written for that purpose, and thus lack appropriate methods descriptions and quality controls that would allow direct adoption for use. Preparing and testing a method for use in the field requires substantial effort and resources. Many of the descriptions of these methods include claims of method efficiency that are contradictory. Thus, it is necessary to develop a procedure to evaluate methods.

There are several models for discerning between methodologies. The US EPA uses an elaborate testing methodology for comparing chemical detection methods. The US EPA also has an established procedure for evaluating methods for detection of bacteria. Neither of these methods, nor those developed for comparing diagnostic methods for human and animal disease, may be directly adopted to evaluate methods for detecting protozoa in water. Chemical, human and animal diagnostic methods are generally designed to detect their targets at higher

concentrations than are protozoan methods for surface or tap water samples. Methods for detecting bacteria generally rely on the geometric increase in bacterial populations within short time periods on appropriate growth media, a situation that does not occur with protozoan transmissible stages. Furthermore, the financial incentive for independent development of these diagnostic methods, historically, has been greater.

A working group from the US EPA has developed a set of criteria that could be used to evaluate methods for detection of protozoa in water (Dufour *et al*, 1999; Lindquist *et al*, 1999). Arising from this effort was an understanding of the critical technical variables for method evaluation. These variables are the two closely related factors of sample, and parasites. Sample variables include the sample size, which must be of sufficiently large size to allow for an effective risk determination, and sample composition. The parasites used must be of reproducible quality, and must be used in realistic concentrations. To minimize the cost for testing of methods a minimum of 10 liters sample volume per test was selected, with a parasite concentration of 10 oocysts per liter. This concentration has been chosen to be as relatively high yet still within the realm of possibility. One of the difficulties in preparing samples of this type was seen as the variability in counting as few as 100 oocysts per sample.

MATERIALS AND METHODS

In all studies, the parasites used were Harley-Moon strain, reared in immunocompromized laboratory mice. Oocysts were harvested over an 18 hour period by collection in water, and purified as previously described by cominution with water, sequential sieving through large to fine mesh sieves, (Cicmanec and Reasoner, 1997) with final purification to near homogeneity through sucrose and cesium chloride density gradients (Kilani and Sekla, 1987). Oocysts were stored in reagent grade water with 100 units of penicillin and 100 µg of streptomycin at 4°C. Initially, oocysts as old as 6 months post-harvest were used. Later, oocyst usage was limited to within two months post-harvest.

This paper will describe three studies undertaken at this laboratory to satisfy the aims of this project. The first was a comparison of 5 methods of aliquoting *C. parvum* oocysts, to determine which produced the least variability while counting given concentrations of oocysts (Bennett *et al*, 1999). The second study was conducted to determine the accuracy of six different methods of delivering 100 oocysts for possible use as a spike for adding to a sample for testing. The final study was an evaluation of 4 different methods

that have been proposed for detection of *Cryptosporidium* spp in surface waters (unpublished observations). This evaluation was conducted using oocysts of *C. parvum*, enumerated with the best method, as demonstrated by the previous studies.

Initial studies to determine variability in oocyst counting methods were as reported in Bennet *et al* (1999). Briefly, each of 5 methods (hemacytometry, epifluorescent well slides, chamber slide, electrostatic particle counting, and flow cytometry) were used to count 20 replicates of each of 4 dilutions of an oocyst suspension. The dilutions were chosen so that at least some of the concentrations fell within the optimal counting range of each method, yet overlapping with dilutions used for other methods. The variance of these counts was plotted against the concentration of oocysts.

To determine the accuracy of the counting methods, six methods were used to enumerate oocysts: hemacytometry, chamber slides, epifluorescence well slides, electrostatic particle counting, solid phase cytometry, and flow cytometry. Stock oocyst suspensions were prepared by enumerating oocysts using a hemacytometer, and diluting volumes of the initial stock suspension to obtain appropriate final concentrations for each method. Concentrations of oocysts in the stock solutions were enumerated by five of the six methods: hemacytometer, chamber slide, epifluorescent well slide, electrostatic particle counter and solid phase cytometry as described elsewhere (Bennett *et al*, 1999). A volume calculated to deliver 100 oocysts was pipetted onto a polycarbonate track etched membrane. The sixth method, flow cytometry, was used to deliver 100 oocysts directly to a membrane. These membranes were stained with fluorescent antibodies and examined by solid phase cytometry, followed by microscopic examination. Six replicates were done for each method. Results of these counts were compared by analysis of variance.

To determine if the technical criteria that had been developed (Lindquist *et al*, 1999) were realistic, these criteria were applied to 4 different direct counting methods of detecting *C. parvum* in water (unpublished observations). Surface and tap water samples of 100 liter volume were taken and paired with reagent water samples, for a total of 7 surface water samples, and one tap water sample. Half of each sample was filtered through 293 mm 1 µm absolute porosity membranes and the filter eluted with a buffer containing surfactant (US EPA, 1999a). The other half of the sample was seeded with flow cytometry enumerated *C. parvum* oocysts at a rate of 10 oocysts per liter, filtered and eluted in the same manner. The filter eluates were split and analyzed such that the equivalent volumes of

10 liters were analyzed by each method. The four methods chosen were a modification of the ICR method (US EPA, 1995) which differed from the ICR method in the fluorescent antibody used, Method 1623 (US EPA, 1999b), solid phase cytometry (Chemunex, Monmouth Junction, New Jersey) and flow cytometry (AusFlow, Australian Environmental Flow Cytometry Group *trading as* Macquarie Research Ltd in collaboration with Australian Water Technologies Ltd, 1999).

Presumptive recoveries from each of these methods were compared by analysis of variance and F² testing to compare both the accuracy and repeatability of these methods. Confirmed results were compared by Student's *t*-tests, due to the absence of confirmed results from two of the methods.

RESULTS

The most reproducible methods with the lowest variability for counting low numbers of oocysts were flow cytometry and electrostatic particle counting. Hemacytometer counting results were acceptable, with less than 10% coefficient of variance if the concentration of the stock being counted was at least approximately 60,000 oocysts per ml. The chamber slide technique failed largely due to drying around the perimeter of the slide, which reduced the maximum countable number of oocysts to below that which would have produced acceptable results. The well slide method was the most variable, possibly due to oocysts washing off the slide during staining in a non-

reproducible manner. The maximum number was also limited by the time required to read these slides.

Flow cytometry was the most accurate of the methods tested for aliquoting 100 oocysts. In the initial analysis of variance, the fluorescent well slide data was so divergent from the other data, that the analysis was skewed away from significance for the other methods. When this method was eliminated from the analysis, the flow cytometric method was found to be most accurate, followed by the solid phase cytometry method. The hemacytometer and electrostatic particle counting methods were indistinguishable in third place. The chamber slide method was the least accurate of these methods (Table 1).

Using spiked reagent water, the solid phase cytometry method had the highest and most reliable recovery followed by flow cytometry, the ICR method and Method 1623. When surface and tap water samples were used, the solid phase cytometry method still had the highest recovery and lowest variability followed by the ICR method, the flow cytometric method and Method 1623 in that order (Table 2, adapted from unpublished observations).

There was no method for confirmation within the flow cytometric method, and the ICR method failed to confirm any of the oocysts by differential interference contrast microscopy. Both the solid phase cytometry method and Method 1623 use 4,6-diamidino 2-phenylindole dihydrochloride (DAPI) staining as a method of confirmation, but with different staining protocols. The solid phase cytometry protocol was able to confirm 4.9 times more oocysts than was Method 1623.

Table 1
Comparative accuracy of methods for enumerating oocysts in suspension.

Method	Mean	Tukey grouping		
Epifluorescence well slide	144	A		
Chamber slide	125	A	B	
Flow cytometer	101		B	C
Solid phase cytometer	96		B	C
Electrostatic particle counter	73			C
Hemacytometer	73			C

Numerical values are means from 6 trials of *C. parvum* oocyst counts by solid phase cytometry with checking of antibody stained oocyst like objects by microscopy, having delivered a presumed 100 oocysts onto a membrane by each of the methods given. Methods with different letters are significantly different by Tukey's groupings following analysis of variance around these means.

Table 2
Evaluation of methods for detecting *Cryptosporidium* spp in various water samples.

Method	\bar{X} (Presumptive) recovery	Methods with significantly different means ($p < 0.05$)	Standard deviation, \log_{10} recovery	Methods with significantly different standard deviation ($p < 0.05$)
Spiked reagent water samples				
Solid phase	31.3	ICR , 1623	0.140	1623
Flow	28.9	1623	0.210	1623
ICR	17.3	Solid ph	0.232	-
1623	14.3	Solid ph, Flow	0.417	Solid ph, ICR
Spiked surface and tap water samples				
Solid phase	48.6	1623	0.206	1623, Flow
ICR	41.2	1623	0.327	Flow
Flow	29.8	-	0.503	Solid ph
1623	11.5	Solid ph, ICR	0.654	Solid ph, ICR

Methods listed in the significant differences columns are significantly different at the $p < 0.05$ level from the method listed in that row. Solid ph = Solid phase. N = 8 reagent water samples, and 7 surface water samples, with 1 tap water sample.

DISCUSSION

It is possible to develop a set of technical criteria, by which methods for detection of protozoa in water may be evaluated. Enumeration of the test organisms and sample matrix type are the most important factors in this process of evaluation. In this study, flow cytometry was the most accurate and reproducible method of providing low numbers (<1,000) of oocysts for seeding into samples to test methods. This is a reasonable conclusion as flow cytometry is capable of precisely enumerating small numbers of oocysts. The other methods used were primarily methods of estimating the density of oocysts in suspension, and error may be introduced in the sampling of these suspensions either during enumeration or delivery of the oocysts, or both.

The solid phase cytometer had the highest and most reproducible recovery of oocysts from both reagent and other water samples. Immunomagnetic separation was used as part of the preparative process for this and several other protocols, and appears to be a good method of separating oocysts from other debris for analysis. The solid phase cytometer makes analysis faster and easier, identifying probable oocysts for confirmation by a microscopist. All the other methods required the microscopist to evaluate an entire slide, a time consuming process. The solid phase cytometer

was not able to analyze samples with large numbers of oocyst like objects. With very large numbers (thousands) of objects on a filter the memory buffer of the cytometer overflowed and the analysis aborted. If there were more than about 500-700 or more presumptive oocyst-like objects to be confirmed, the analysis also failed. This was because confirmation of this large number of presumptive oocyst-like objects required so much time, that the filter would dry out during the process, curling and preventing completion of the microscopic evaluation.

Differential interference microscopy was not an effective means of confirmation of oocyst like objects as oocysts. Identification of 4 nuclei within an oocyst by their characteristic DAPI staining was a more reliable method.

Automation introduced to the examination of water samples lowers the variability of the analysis, and increases the overall efficiency. Still, none of the methods tested had more than 50% recovery of the spiked oocysts. Also, none of the methods tested addressed the issue of species within the genus *Cryptosporidium* spp, or the issues of strain, viability, infectivity or pathogenicity of the organisms detected. As more tests are conducted for more of these variables, it is expected that concentration estimates of organisms in the environment will decrease disproportionately to their true presence.

ACKNOWLEDGEMENTS

The authors would like to sincerely thank James Owens from the National Risk Management Research Laboratory, US EPA, Cincinnati, OH, for generously supplying oocysts for this study. Thanks are also due Dr Andrew Campbell, Dynal Corporation for generously providing immunomagnetic beads and reagents used throughout this study.

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