Detection of Infectious Cryptosporidium in Conventionally Treated Drinking Water [Project #3021]

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OBJECTIVES:
The primary objective of the project was to determine the prevalence of infectious Cryptosporidium spp. in conventionally treated drinking water. The research covered four specific objectives: (1) assess the recovery efficiency of a modified version of USEPA Method 1623 with seeded 1,000 L finished water samples; (2) compare the sensitivity and reproducibility of three cell culture based Cryptosporidium infectivity assays; (3) evaluate the suitability of all methods for genotyping infectious oocysts; and (4) use the most effective method for a nationwide survey of infectious Cryptosporidium oocysts in large volume samples of drinking water.

BACKGROUND:
Almost two decades after the Milwaukee Cryptosporidium incident, outbreaks of cryptosporidiosis still occur, linked to both drinking water and recreational water. Research studies report high frequencies of Cryptosporidium oocyst detection in untreated and finished drinking water, although monitoring programs typically demonstrate lower occurrence. Oocysts are resistant to chlorine disinfection at the concentrations typically applied during drinking water treatment. However, correctly operating treatment plants that utilize filtration usually remove oocysts from source water with high efficiency. Nevertheless, oocysts have been detected in up to 40% of treated drinking water samples at concentrations as high as 0.5 oocysts/L.

The results of plant influent monitoring under the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) will determine whether water utilities need to install additional treatment based on average Cryptosporidium oocyst concentrations in their source waters. Most utilities are likely to be placed in Bin 1 (<0.075 oocysts/L), requiring no additional treatment. However, there is a lot of uncertainty in estimating the contribution of drinking water to the nationwide cryptosporidiosis burden. According to the only study on the prevalence of infectious Cryptosporidium oocysts in conventionally filtered drinking water in the United States, 1.4% of finished water samples contained infectious oocysts, and 27% of surface water treatment plants released infectious oocysts in their finished water at least once during the study period. The results translated to an annual cryptosporidiosis risk of 52 infections per 10,000 people, which is much higher than the 1 in 10,000 annual risk of infection goal set by the USEPA. These data indicate that public health may be compromised by municipal drinking water. Consequently, the current project aimed to assess the repeatability of the previously published study on the prevalence of infectious oocysts.
APPROACH:
This study compared three assays for detecting Cryptosporidium spp. infections in cell culture. The assays were: (1) immunofluorescence microscopy (IFA), (2) a polymerase chain reaction targeting Cryptosporidium spp.-specific DNA (PCR), and (3) reverse transcriptase-PCR targeting Cryptosporidium spp.-specific messenger RNA (RT-PCR). Human cell monolayers (HCT-8 cells), grown in either 8-well chamber slides or 96-well plates, were inoculated with a variety of oocysts to assess assay performance. Method evaluation included live oocysts enumerated by flow cytometry, blind-spiked samples, and oocysts that were inactivated by heat, gamma-irradiation, and ultraviolet radiation (UV). Factors used to determine the most effective method were sensitivity, intra- and inter-laboratory reproducibility, frequency of false-positives, robustness, and ease of use.

The survey of large volume samples from 14 drinking water treatment plants across the United States (average volume = 943 L) used the most effective of the three detection assays to assess the prevalence of infectious oocysts. Sample collection, filtration through Envirochek HV capsules, elution, and immunomagnetic purification followed a modified version of USEPA Method 1623. The modification involved soaking the filter in 5% sodium hexametaphosphate prior to eluting oocysts. Recovery efficiencies were evaluated with 1,000 L matrix samples spiked with freshly shed oocysts and gamma-irradiated ColorSeed oocysts. Two laboratories performed infectivity assays for most of the finished water samples.

Infections detected by PCR and RT-PCR could be genotyped directly by sequencing primary amplification products or by amplifying and sequencing secondary target genes. In addition, the project developed a method for genotyping infections detected by IFA. The method involved lysing a focus of life stages on the IFA slide and then removing the lysate to a tube for subsequent DNA extraction, PCR, and sequencing.

RESULTS/CONCLUSIONS:
All assays detected infection of cell cultures with low numbers of flow-cytometry enumerated oocysts, including infection with a single oocyst. Based on both qualitative and quantitative comparisons, the cell culture (CC)-IFA method was selected as most effective for assessing the occurrence of infectious oocysts in finished drinking water. It consistently detected infections with three oocysts or less, generated few false-positives (all of which could be discounted by an experienced microscopist), was reproducible and relatively simple to perform. Applying the entire CC-IFA-genotyping method to naturally occurring infectious oocysts in wastewater demonstrated its suitability for environmental monitoring.

Fourteen treatment plants across the United States participated in the survey of infectious oocysts in finished water with sample volumes ranging from 83.5 to 2,282 L and an average of 943 L. The survey analyzed 370 samples totaling 349,053 L of treated drinking water. The volume of water filtered for each sample depended on water quality characteristics, the amount of water passing through the filter before it clogged, problems with individual filtration rigs (e.g., inadequate pressure or flow control), or operational issues at the treatment plant. Nevertheless, 90% of samples were >600 L and 82% were
>900 L. Most plants provided monthly or biweekly samples for two years, although some of the plants only collected samples for part of each year.

The average recovery efficiency for 1,000 L samples of finished drinking water spiked with gamma-irradiated EasySeed oocysts and analyzed by both laboratories was 71% \((n=10)\). The average recovery efficiency for samples from all participating utilities spiked with ColorSeed oocysts was 42% \((n=45)\).

None of the 370 finished water samples produced infections that were detected by the CC-IFA assay. Control infections and matrix spike samples demonstrated that oocyst recovery procedures and the infectivity assay performed as expected. Based on a previously published risk assessment calculation and a total analyzed volume of 349,053 L, the lack of positives in the current study translates to an annual risk of less than one infection per 10,000 people.

**APPLICATIONS/RECOMMENDATIONS:**

*Cryptosporidium* spp. oocysts are resistant to chlorine disinfection at the concentrations typically applied in drinking water treatment plants. Although correctly operating treatment plants that use filtration usually remove oocysts from water with high efficiency, low levels of *Cryptosporidium* oocysts occur in finished drinking water. Current monitoring programs using Method 1623 will provide oocyst occurrence data for untreated source waters but will not provide information on oocysts in finished water or assess the infectivity of detected oocysts. Therefore, it will still be difficult to assess the actual public health risk posed by *Cryptosporidium* in drinking water.

This project applied a standardized cell culture assay to environmentally-relevant low numbers of oocysts recovered from large volumes of finished water using a modified version of Method 1623. The cell culture method involved incubating inoculated HCT-8 cells in 8-well chamber slides at 37°C for 64–72 hours, staining with anti-sporozoite antibody and a FITC-labeled secondary antibody, and enumerating infections by epifluorescence microscopy. Widespread application of this method to finished water will allow a more accurate assessment, with increased confidence, of the public health significance of *Cryptosporidium* oocysts in drinking water.

The inter-laboratory method comparisons demonstrated that the CC-IFA method is suitable for monitoring infectious *Cryptosporidium* in finished water. Information on the relative sensitivity of the methods and their rates of false-positive detections will allow other investigators and utilities to make a more informed decision when selecting a method for either routine monitoring or stand-alone research studies.

The project highlighted the difficulty in applying a non-compliance microbiological method when the results could have adverse legal, operational, public health, and public relations consequences for participating utilities. Many utilities were reluctant to participate because of concerns over the possible consequences of detecting infectious oocysts in their finished drinking water. Consequently, the majority of utilities
participating in this study had low levels of *Cryptosporidium* in their source waters and so detecting infectious *Cryptosporidium* in their finished water was unlikely.

Since a broader range of utilities may need to be surveyed for the presence of infectious *Cryptosporidium* oocysts, the second round of *Cryptosporidium* monitoring under the LT2ESWTR should include infectivity analyses on finished water. Mandatory infectivity analyses would not be practical for all utilities but a subset of utilities could be monitored on a relatively frequent basis. The cell culture method is sufficiently developed and standardized that the laboratory capacity could be readily built within the regulatory timeframe. Options for implementing cell culture-based infectivity monitoring include (1) on-site cell culture facilities at utility laboratories, (2) purchasing ready-to-use cell monolayers from a commercial supplier and then performing oocyst recovery and infectivity assay procedures in-house, and (3) shipping recovered oocysts to a centralized cell culture testing facility.

**Specific Project Recommendations**

1. Implement monitoring for infectious *Cryptosporidium* oocysts in finished water using a standardized cell culture assay. These assays may be carried out using in-house facilities or contract laboratories.
2. Conduct follow-up studies that include state public health professionals and federal regulators as part of the project team. This expanded team may help to reduce the reluctance of utilities to participate.
3. Focus future surveys on Bin 2 or higher utilities rather than attempting to capture a national average risk of infection. Bin 2 and higher utilities represent an increased risk of infection compared to the majority of plants, which will be classified as Bin 1. Surveys could include intensive sampling of a few plants over an extended period.
4. Optimize the *Cryptosporidium* cell culture method, to increase proportional infectivity, which will increase the likelihood of detecting infection with a single oocyst.
5. Assess the range of *Cryptosporidium* species and genotypes that can infect HCT-8 cells and the specificity of the anti-sporozoite antibody to infectious stages of species other than *C. parvum*, *C. hominis*, and *C. meleagridis*.

**PARTICIPANTS:**
This project could not have been possible without the generous support and participation of the 14 anonymous utilities that provided filtered water samples throughout the study.