United States Environmental Protection Agency **Office of Research and Development**

National Exposure Research Laboratory
Research Abstract

Government Performance Results Act (GPRA) Goal # 2.3.2
Annual Performance Measure # 206

Significant Research Findings:

Improved method(s) for CCL-related microbes for use in Unregulated Contaminant Monitoring Rule (UCMR)

Scientific Problem and Policy Issues

Many human pathogenic viruses can cause disease in individuals who are exposed to inadequately treated drinking water or to recreational waters contaminated with fecal waste. For example, enteroviruses are commonly found in U.S. surface waters and can cause a range of diseases including diabetes, encephalitis, eye infections, gastroenteritis, hepatitis, meningitis, myocarditis, and paralysis. Similarly, caliciviruses and rotaviruses cause gastroenteritis and can be transmitted by water. The Unregulated Contaminant Monitoring Rule (UCMR) was established to obtain occurrence data so the Office of Water can assess risk and make regulatory determinations for microbes and chemicals on the Contaminant Candidate List (CCL). To date, however, waterborne viruses have not been included in the UCMR due to the lack of validated methods to detect a broad range of these pathogens.

Research Approach

This research abstract describes three studies designed to test virus method improvements and performance over a range of water types. Specifically, these studies describe (1) the use of EPA methods to detect waterborne virus during an outbreak, (2) the development and testing of an assay that is able to detect a wide variety of viruses, and (3) the design of internal controls that are able to more easily detect when measurements of viruses by polymerase chain reaction (PCR) are likely to be inhibited and, therefore, be artificially low.

The first study field tested EPA's method to detect caliciviruses in outbreak settings (Anderson *et al.*, 2003). Scientists from the National Exposure Research Laboratory worked with those from EPA Region VIII, the Centers for Disease Control and the Wyoming Department of Health to investigate a drinking water outbreak that occurred in central Wyoming. Viruses were collected from the outbreak-associated drinking water source by concentrating a sample of 2,000 liters on a positively charged cartridge filter. Eluted virus was measured using a polymerase chain reaction (PCR) technique designed to detect the majority of noroviruses, which are the group of caliciviruses that are known to cause waterborne disease outbreaks.

The second study tested both the behavior of a molecular method for enteroviruses, hepatitis A virus, noroviruses, reoviruses, and rotaviruses in a variety of surface water types and the benefit of a control for the measurement of

virus recovery. The study evaluated whether or not a method that had been designed for detecting viruses in groundwaters would also be useful for detecting viruses in more turbid surface waters. This was done by testing surface water sites that are part of the U.S. Geological Survey's National Water Quality Assessment (NAWQA) Program. Each of five sites was tested three times with one site being sampled after a rain event when the streams would contain higher levels of sewage, organic material and minerals.

The study also evaluated the efficiency with which the sample collection methods are able to collect viruses from real world water samples. When viruses are collected from ground and surface waters by filtration through positively charged filters, the recovery rates may be severely impacted by water quality factors, such as mineral content (e.g., negatively charged ions of salts), organic compounds (e.g., humic acids) and pH. However, since measurement of recovery in the field is rarely reported, interpreting virus occurrence data is difficult. Recovery studies are not done routinely because of the difficulty in both transporting live virus into the field without loss of infectivity and in preventing the seed virus from contaminating the environment. In this study, virus recovery was determined by collecting unseeded and virus-seeded samples at each sampling event using a procedure that was simple and that did not contaminate the environment. This was done by injecting a vial of Sabin poliovirus vaccine stock into the water before the filter and by collecting the water that passed through the filter into a metal drum containing disinfectant.

The third study developed internal control standards that can be used to test for PCR inhibition. Environmental waters often contain chemicals that can inhibit PCR and produce false-negative PCR results. Methods to detect viruses in water normally contain procedures to reduce the inhibition, but these are not equally effective in all water types. Due to this variability, all samples tested by PCR must include a control to demonstrate whether or not inhibitors are removed. This control is typically a virus-seeded sample; however, this requires that each sample be analyzed twice. Internal control standards reduce labor and reagent costs by allowing the control to be analyzed along with viruses of interest in the same reaction tube. The internal standards designed in this study are RNA molecules that are identical to the viruses being detected, except for a small modification in the region used to detect the virus through PCR amplification. The modification is designed to produce a PCR product that is smaller than that from the parent virus genome. This allows the amplified internal control to be distinguished from the amplified virus product by electrophoresis. The internal standards are made by genetically modifying PCR fragments and cloning them into DNA expression vectors. The expression vectors have sequences that can be used to produce large quantities of RNA molecules, which are then purified and used as the internal control standards.

Results and Impact

The first study confirmed the utility of EPA's method for detecting noroviruses in drinking water during an outbreak investigation. An outbreak of gastroenteritis affecting at least 84 patrons had occurred in a saloon in central Wyoming during September and October, 2001. The investigation demonstrated that the ground water well being used by the saloon was impacted by septic system sewage and that a water chlorinator had failed during the time of the outbreak. Water collected

from the well was positive for fecal coliforms. It also contained the same strain of norovirus that was found in patients affected from the outbreak.

The second study showed that an EPA molecular method designed for virus detection in ground water could be used with surface water. NAQWA sites in Washington State, Iowa, Ohio, West Virginia and South Carolina were selected based upon geographic location, population density and land usage (agricultural, mining, urban, etc.). Population densities of the chosen sites ranged from 16 to 15,540 individuals per square kilometer. Water quality parameters were typical for streams around the US. Water temperatures ranged from 0 to 12.6°C, reflecting the winter and spring sampling conditions, and pH values ranged from 5.4 to 8.1. Turbidity ranged from 0.7 to 344 nephelometric turbidity units and *E. coli* concentrations ranged from 59 to 23,000 colony-forming units per 100 mL. Each site was tested three times for viruses using cultural and molecular methods. All sites were positive for viruses by both methods. A total of 87% of the samples were positive for enteroviruses, 30% for reoviruses, 20% for rotaviruses and 17% for hepatitis A virus. Quality control data were critical in the interpretation of the molecular results. Based in part on the results from this study, a number of quality controls that are not normally considered were recommended for future virus occurrence studies, and these recommendations have been incorporated into a joint Office of Water/Office of Research and Development guidance document entitled, "Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples." Infectious enteroviruses were found in four samples collected from streams which would have met EPA's guidelines for safe recreational water usage. This suggests that indicator levels that are at or below the guidelines do not always predict the viral quality of waters.

This study also demonstrated that the procedure to measure virus recoveries could be performed safely in the field. Poliovirus recoveries from stream water averaged 45% and ranged from 16 to 65%. Large recovery differences were observed between individual samples at two of the sites and recovery rates did not appear to be inversely correlated to turbidity as generally assumed. Unfortunately, with the limited number of samples in this study, determining specific correlations between water quality and virus recovery was not possible. However, the importance of virus recovery controls was suggested by the detection of a greater number of virus types in all samples with higher virus recoveries, with the possible exception of samples from the West Virginia site.

The third study describes the development and testing of internal controls for enteroviruses, hepatitis A virus (HAV), Norwalk virus and rotaviruses. The controls were shown by gene sequencing to have only small differences between the size and the sequence of the control and that of the respective virus. It was shown that each control could be distinguished from the virus for which it was designed by electrophoresis and by hybridization with specific probes. Procedures to produce internal standards comprised of RNA (free of DNA from the expression vector) were developed and were successfully used to demonstrate the absence of inhibitors from environmental water samples. These controls not only reduce labor and reagent costs, but they also are thought to decrease the number of false positive reactions by eliminating the need to run virus-seeded samples.

	The results of this research support the Government Performance and Results Act Goal 2 ("Clean and Safe Water"), Sub-Objective 2.3.2 ("Conduct Leading-Edge Research"), and Long Term Goal DW-3 ("Unregulated Contaminants and Innovative Approaches, By FY 2010, develop new data, innovative tools and improved technologies to support decision making by the Office of Water on the Contaminant Candidate List and other regulatory issues, and implementation of rules by states, local authorities and water utilities"). The research was done in support of an FY05 annual performance goal #131 ("Provide the Office of Water with the results of health effects, exposure / methods and treatment studies, in support of decisions to regulate or not regulate at least five pathogens and toxins on the Contaminant Candidate List.") and annual performance measure #206 ("Improved method(s) for CCL-related microbes for use in Unregulated Contaminate Monitoring Rule (UCMR), e.g. enteroviruses, caliciviruses, rotaviruses").
Research Collaboration and Research Products	A portion of this research was performed under an Interagency Agreement with the U.S. Geological Survey. The findings of this research have been published (Publication No. NERL-CI-MCEARD-03-003, NERL-CI-MCEARD-03-032, NERL-CI-MCEARD-03-092, respectively): Parshionikar, S.U., Willian-True, S., Fout, G.S., Robbins, D.E., Seys, S.A., Cassady, J.D., and Harris, R. "Waterborne outbreak of gastroenteritis associated with a norovirus." *Appl. Environ. Microbiol*. 69:5263-8, 2003. Denis-Mize, K., Fout, G.S., Dahling, D.R., and Francy, D.S. "Detection of Human Enteric Viruses in Stream Water with PCR and Cell Culture." *J. Water Health*. 2:37-47, 2004. Parshionikar, S.U. Cashdollar, J., and Fout, G.S. "Development of homologous viral internal controls for use in RT-PCR assays of waterborne enteric viruses." J. Virol. Methods, 121: 39-48, 2004.
Future Research	Additional improvements are still needed before the methods can be used for routine water monitoring for viruses. The highest priority areas for needed improvements are the development of an alternative to the expensive positively charged 1MDS filter and the development of techniques to define human health risks based upon molecular assays.
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