Workshop to Develop a Protocol for Reliable Genetic Methods for the Detection of Viruses, for Use in EPA's Water Programs

January 15-16, 2003

U.S. Environmental Protection Agency
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Summary

The U.S. Environmental Protection Agency (USEPA) and the American Water Works Association Research Foundation (AWWARF) held an expert workshop in Cincinnati, Ohio on January 15-16, 2003 to explore ways to standardize polymerase chain reaction (PCR) methods for detecting waterborne pathogens. The workshop announcement, prospectus, and agenda were provided to all participants. Invited speakers were asked to present their research on: applications of PCR for virus detection in water; the quality assurance (QA) and quality control (QC) measures employed with molecular methods to ensure their reliable performance; and the use of molecular methods for assessing health risks from drinking water exposures. Following the presentations, a panel provided independent review and commentary of the presentations, and offered opinions regarding the feasibility of adopting PCR methods for occurrence and health effects studies.

The goals of the workshop were to:

- Evaluate the feasibility of using PCR for monitoring the presence of viruses in water.
- Identify standard QA/QC techniques (e.g., positive and negative controls, internal standards, work area set-up) for increasing the reliability of PCR-based method results.
- Identify one or more PCR method that can be standardized for validation studies.
- Determine how PCR might be used in risk assessment activities. Specifically, can current PCR methods be used to determine pathogen viability and infectivity? If not, can methods be modified to allow this determination?
- Identify areas of research that might lead to improvements in molecular detection methods for waterborne pathogens.

To help meet the goals and objectives of the workshop, speakers who presented a PCR-based method were asked to answer a list of 20 questions designed to explore the robustness of the method presented, and to facilitate comparisons between methods. The questions were also aimed at selecting elements that could be incorporated into a consensus method, which could then be subjected to

validation studies. The responses for each method were provided to all participants.

DAY 1

Introductions

George Hallberg, The Cadmus Group, Inc.
Dr. Hallberg facilitated the meeting, and introduced Mr. Gregory Carroll, Chief, Technical Support Center (TSC), Office of Ground Water and Drinking Water (OGWDW), USEPA, who welcomed the participants to Cincinnati. Dr. Gerard Stelma, Acting Director of the Microbiological and Chemical Exposure Assessment Research Division, National Exposure Research Laboratory (NERL), USEPA, provided an overview of the Office of Research and Development, and their activities in risk assessment, risk management, and risk exposure. He emphasized the need for pathogen monitoring to acquire and assess environmental occurrence and health effects information.

Background and Purpose

Keya Sen, OGWDW, TSC
Dr. Sen reviewed the history of PCR methods and the limitations of the methods which have precluded their use in assessments of pathogen occurrence. Pathogen detection studies using PCR and culture methods in parallel have produced widely differing results, casting doubt on the reliability of PCR data. Potential limitations of PCR-based methods include: the presence of inhibitors of PCR in environmental waters; the susceptibility to contamination from positive samples; and the inability to determine viability/infectivity. Use of culture methods as a 'gold standard' upon which to evaluate PCR is limited, because some viruses have not been successfully propagated in cell culture. Among the potential advantages of PCR are its ability to detect viruses that cannot be cultured or which are difficult to culture using known cell lines, the ability to produce results in hours instead of weeks, and high sensitivity.

Shay Fout, National Environmental Research Laboratory (NERL)
Dr. Fout reviewed the general properties of viruses that cause waterborne diseases. He pointed out that virus detection methods that work well in the laboratory often do not work well on field samples. He reviewed the virus groups that are known causes of waterborne outbreaks, including: hepatitis A, noroviruses, polioviruses and rotaviruses, as well as virus groups that have the potential to cause waterborne diseases, including non-poliovirus members of the enterovirus group, sapoviruses, astroviruses, reoviruses, and enteric adenoviruses 40/41. He reviewed some of the epidemiological data for these viruses that have linked viral outbreaks to a water route, the diseases they were capable of causing, and the special control problems some of the viruses may have, including resistance to common disinfection practices.

Presentations

Mark Sobsey, University of North Carolina

Dr. Sobsey gave an overview of the use of molecular techniques for detection of waterborne viruses, emphasizing challenges ranging from sample collection, to use of PCR data for risk assessment. He stressed that low numbers of viruses are present in environmental samples, necessitating collection of large sample volumes for analysis to ensure sensitivity. The sample must then be concentrated, at the same time minimizing inhibitory effects of matrix contaminants. The tendency of viruses to aggregate and adsorb to particles in the environment complicates the design of statistically significant sampling plans, and adds considerable expense to pathogen occurrence studies. Dr. Sobsey spoke about a major problem with the interpretation of molecular results. Molecular methods detect virus particles that are both 'alive' and 'dead'. Dead particles do not pose health risks, but are an indicator of vulnerability to contamination. He suggested several potential ways in which virus viability and infectivity could be measured especially where the viruses were not culturable and recommended future research in that direction. He suggested that approaches that target the mRNA of DNA viruses, and negative strand RNA of positive strand RNA viruses, or that use cell receptors or other ligands to capture virus, could be used to determine virus viability. He showed data indicating that the cell receptor approach could be used for further concentration and purification of viruses prior to analysis by a molecular method. Because virus particles killed by disinfection do not bind to the receptors, they are not detected in the molecular assay. Dr. Sobsey proposed using culturable viruses or bacteriophages as indicators of fecal pollution of ground water, but acknowledged that improvements in cell culture technology were necessary before virus monitoring could become practical. He listed a series of priorities, issues and challenges regarding developmental aspects of molecular methods for virus detection, and made recommendations on approaches, priorities, and goals for short and long term consideration. For the immediate short term, he recommended the use of two approaches: direct molecular detection for non-culturable viruses; cell culture plus molecular detect for viruses which were abundant in water and for which the most well developed molecular reagents were available. For determining infectivity of the viruses, he suggested exposing sample concentrates to proteolytic enzymes to which intact viruses were resistant. When cell culture plus molecular detection was going to be used, he asked that the best cell lines, capable of detecting the most enteric viruses, be chosen and optimized for maximum infectivity. He stressed the use of internal controls and sequencing of the isolates. He urged more collaboration among investigators to arrive at consensus methods.

David Battigelli

Dr. Battigelli discussed the detection of viruses in five studies on ground water from the states of Maryland, Minnesota and Wisconsin. Viruses were concentrated on 1MDS filters, eluted from filters and reconcentrated by organic flocculation using the standard procedure defined by EPA's Information Collection Rule (ICR). RNA was released from viruses in the concentrated eluate using guanidium isothiocynate (GITC) and phenol-chloroform. RNA was further purified on Sephadex G100 columns and then treated with Chelex 100. Samples were assayed by a large volume RT-PCR procedure for enteroviruses, hepatitis A virus, rotavirus, and Norwalk-like viruses. The optimal conditions for each virus group was presented. He found that 5-10% of the samples could not be assayed because of the presence of inhibitors. He also described an optional RNA precipitation step, that increased the

sensitivity of the assay 15 fold. However, the process increased the assay time and did not solve the problem of inhibitors. He stressed the need for a confirmatory step following PCR-agarose gel electrophoresis such as Southern hybridization. Overall, Dr. Battigelli found that 6% of the sites were virus positive by RT-PCR while less than 1% were positive by the ICR cultural method. He stated that considerable training efforts were required to establish a PCR testing laboratory and the associated expense was a potential limitation to its widespread application.

Shay Fout, NERL

Dr. Fout presented an RT-PCR method that was developed at EPA. The initial portion of this method uses the ICR sampling procedure to concentrate viruses on 1MDS positively charged filters. Virus particles are then eluted from filters with a non-flocculating beef extract and reconcentrated with celite (diatoms). Dr. Fout reported virus recovery from seeded lab samples of 75% ± 35% using the adsorption, elution, and reconcentration procedures from the ICR method, and 95% ± 2% using the celite procedure. The molecular method includes a three step procedure to remove environmental inhibitors of PCR. Virus particles in the concentrated eluate were centrifuged through a 30% sucrose pad, treated with dithiozone and 8-hydroxyquinoline in chloroform, butanol, methanol and trichloroethane, and then further concentrated to 40 :L on a concentrator that removes substances which are less than 100,000 daltons. The inhibitor removal procedure results in a 74% recovery of seeded poliovirus. The entire method results in a million-fold concentration of the virus particles present in a water sample in which 200L are passed through a 1MDS filter. Dr. Fout then described a multiplex RT-PCR procedure for analysis of enteroviruses, reoviruses, and rotaviruses in one assay, and hepatitis A virus and Norwalk virus in another assay. The complete method was used to study 321 groundwater samples from 29 sites. Enteroviruses, reoviruses, hepatitis A virus, and Norwalk virus were detected in 16% of the samples, and 72% of the sites were positive for viruses by PCR, but the results did not correlate with cell culture results. Dr. Fout then presented the results of two investigations of waterborne disease outbreaks involving noroviruses. The method was used to assay groundwater sources from each outbreak. In both outbreaks a Norwalk-like virus was detected and shown to be identical to viruses found in patients who were sick during the outbreaks. The viruses detected from two different outbreaks belonged to different Norwalk-like virus genogroups. He next recommended a number of QA/QC controls to ensure the quality of PCR data. He provided data to show that virus recovery varies significantly in different water sources. Therefore, he recommended that virus recovery controls be used. Other recommended controls included several negative controls, and equipment blanks to detect nucleic acid contamination (i.e., potentially false positive results). Virus positive controls and matrix spikes were recommended to ensure that the PCR reactions were successful (i.e., to detect potentially false negative results that could be due to missing reagents or the carryover of environmental inhibitors). Hybridization positive and negative controls were included to demonstrate the effectiveness of this technique. Significant laboratory space is necessary to physically separate the different stages in a PCR process, so that adequate QA/QC of the assay is maintained. This was illustrated by Dr. Fout through a flow-chart diagram, and by visits to his laboratories by the workshop attendees at the end of the day's talks. He stated that additional viral occurrence studies were needed to get the true picture of viral contamination. He believes existing cultural and molecular virus detection methods need to be improved and optimized.

Robert Atmar, Baylor College of Medicine

Dr. Atmar presented his research on the use of immunomagnetic capture (IMC)/RT-PCR/enzyme immunoassay (EIA) for detecting noroviruses in environmental samples. He reviewed the *Caliciviridae* family, consisting of the genera *Norovirus, Vesivirus, Sapovirus*, and *Lagovirus*. The noroviruses are divided into two major genogroups, I and II, and cannot be grown in cell culture. Thus, current detection methods rely on RT-PCR assays. Because standard RT-PCR assays are not quantitative, he used a "most probable number" approach to measure the efficiency of the IMC method. The efficiency depends upon the availability of antibodies that recognize a broad range of norovirus strains. He tested several polyclonal and monoclonal antibodies to capture different strains, including some monoclonal antibodies that are broadly reactive with a number of strains. He found that polyclonal antibodies produced better recoveries than monoclonal antibodies for both genogroup I and II norovirus strains. Additional broadly reactive antibodies are needed for further evaluation. He also spoke about the possible use of blood group antigens for concentrating noroviruses, but stated preliminary studies suggest that recovery efficiency is less than that of antisera. Dr. Atmar's RT-PCR assay used rTth polymerase to perform both the activities of reverse transcription and DNA amplification in a single tube. However, this enzyme did not work for some norovirus-specific primer sets. He showed that the confirmation of RT-PCR results with an EIA can be performed more quickly and with similar sensitivities to standard hybridization tests and concluded that IMC/RT-PCR could be a useful strategy for the detection of noroviruses.

Kellogg Schwab, Johns Hopkins University

Dr. Schwab began with a discussion of the importance of QA/QC in PCR assays. He stated that a major difficulty of PCR reactions is the need to concentrate samples of 100L or more to a final volume of less than 100 : L, while removing PCR inhibitors to eliminate false negative reactions. He stressed the importance of controls, such as: the physical separation of sample preparation and PCR testing locations; the use of dedicated, aliquoted reagents, equipment, and lab coats; and rules on not processing field samples after working with PCR amplicons, virus stocks or clones containing viral DNA. These controls minimize the possibility of cross-contamination that leads to false positive tests. He stated that PCR products should be confirmed by sequencing, hybridization, or nested PCR. Dr. Schwab emphasized that any violation of rigid QA/QC standards, or any compromise of the PCR laboratory suite can invalidate laboratory results. He described the sample processing procedure used by his laboratory. The procedure uses concentration of the filter eluate by polyethylene glycol or immunomagnetic separation, and extraction of viral nucleic acid with guanidinium/phenol/chloroform, which can be followed by silica purification. He described his single tube RT-PCR assay that uses the uracil-DNA glycosylase system to degrade previously amplified contaminants and an internal standard to control for PCR inhibition. He stated that it is unwise to use the stock virus for which the assay was designed as a positive control. He also cautioned that the use of internal standards be monitored carefully so that amplification of the target genome was not ablated by competition with the internal standard. He believes that his approach of using a single tube (with a single enzyme or multiple enzymes) for reverse transcription and amplification can greatly improve specificity and sensitivity and prevent laboratory contamination. Dr. Schwab described an assay for detecting Norovirus in surface water that concentrated and purified viral RNA using the procedure described above. Viral RNA was subsequently amplified by single round RT-PCR and amplicons were detected using a DNA

hybridization immunoassay (EIA). The EIA reduces the confirmatory step from 1-2 days to 3 hours and has the potential to be automated. He also emphasized the importance of a healthy skepticism when interpreting PCR results, suggesting researchers ask "yes, but..." questions. He believes that nested PCR should be used with great caution in occurrence or health effects monitoring due to greater potential contamination problems. In addition, the consistently improving sensitivity and specificity being obtained using single round RT-PCR followed by oligoprobe confirmation lessens the need for the use of nested approaches.

Aaron Margolin, University of New Hampshire

Dr. Margolin described the problems associated with traditional cell culture and direct PCR methods. A major problem with cell culture procedures is that they do not detect viruses that fail to cause cytopathic effects (CPE). A major problem with direct PCR is that it does not differentiate between infectious and non-infectious virus particles. He described his experience with an integrated cell culture-nested PCR (ICC-nPCR) that can detect viruses that do not produce CPE without detecting non-infectious particles. He also described the extraordinary facilities and operational quality controls required to obtain reliable results with ICC-nPCR. The facilities included a specially designed PCR suite that had assay components separated into four laboratories. The facilities included a clean room that was operated under positive pressure to reduce the possibility of nucleic acid contamination. The laboratory developed and rigidly enforced controls from a detailed quality assurance manual. These included the defined use of biosafety cabinets, dedicated equipment, the use of positive displacement pipettors, a number of negative quality controls, and a requirement that all positive sample results be repeatable. Dr. Margolin described the ICC-nPCR technique in which BGM and CaCo-2 cell lines were infected with a water sample concentrate for approximately 3-5 days. At this time the cells were lysed and the lysates tested for adenoviruses, astroviruses, enteroviruses and rotaviruses using direct-PCR with specific primers. This was followed by a nested PCR with internal primers. He stated that nested PCR is an exquisitely sensitive assay, and that without the second PCR a lot of samples would have been negative. ICC-nPCR also overcomes the need for extensive virus purification processes to remove environmental inhibitors. Positive results from the nPCR assay were considered to be from infectious virus particles if it was demonstrated that a cell culture lysate tested immediately after virus adsorption was negative or if a true negative result could be demonstrated using sample concentrate diluted to the same ratio as the cell culture inoculum. Four studies were conducted to illustrate the utility of the ICC-nPCR method for various sample matrices, including: samples of biosolids intended for land application; archived samples that had originally been tested for viruses during EPA's Information Collection Rule (ICR); and marine waters. He showed that the method was capable of detecting many more positive samples than could be detected by cell culture alone and that the method does detect viruses that do not produce CPE on cell lines. Dr. Margolin reminded the group that nested PCR provides increased sensitivity and specificity over conventional PCR, but requires stringent QA/QC measures, specially qualified and trained technologists, dedicated equipment, and state of the art laboratory design, all of which can prove to be costly. He concluded by stressing the need for genetically modified positive controls.

Yong Seok Jeong, Kyung Hee University, Korea

Dr. Jeong discussed the use of the total culturable virus assay (TCVA) from EPA's ICR, and standard PCR and ICC-nPCR for surveying waterborne viruses in Korea. The appropriateness of the assay

method and the legal status of results of virus occurrence studies in drinking water are serious issues in Korea. For standard and ICC-PCR, Dr. Jeong used a multiplex, nested PCR format for enteroviruses, adenoviruses, hepatitis A virus and rotaviruses. Culturable viruses were found in approximately 38% of surface and source water samples, 4% of finished water samples at the plant and 3% of tap water samples by the TCVA assay in six studies, while in one study using ICC-nPCR 65% of tap water samples were positive. Dr. Jeong showed the results of direct comparisons of samples assayed by the TVCA and the ICC-nPCR methods and by direct PCR and ICC-nPCR methods. As a result of these studies, he concluded that the ICC- nPCR- assay is not necessarily more sensitive than the TVCA assay, especially when the specific primer sets for various virus sets were restricted, and that direct PCR was the least sensitive in the whole study. Cell culture assays, on the other hand, may underestimate virus occurrence and results are not available for days to weeks. PCR-based assays may eventually supercede cell culture assays when problems of false positives, quantification, and cost are resolved. Dr. Jeong also presented data on using and validating a DNA-chip based confirmation approach as an alternative to hybridization for enteroviruses, adenoviruses and rotaviruses. Oligonucleotide chip-based methods offer great potential for increasing throughput, and reducing cost through automation. Dr. Jeong cautioned that a limitation of PCR-based assays is that primers are based upon known virus sequences, and may not detect emerging viruses, thus raising a question of how to interpret a negative assay result.

George Di Giovanni, Texas A&M University

Dr. Di Giovanni presented his research on developing an 8-hour method to detect infectious virus particles in wastewater using real-time quantitative PCR based upon the TaqMan system. Viruses present in wastewater were collected onto 47-mm 1MDS capsule filters. The capsules were eluted with beef extract and then the eluate was concentrated to 100 -150 : L by using 100,000 molecular weight cutoff filtration. A "host cell capture" assay using BGM cell monolayers was developed to remove non-infectious virus particles from a sample. This assay is based upon a hypothesis that non-infectious virus particles will not bind specifically to host receptors and that these particles can be washed off cell monolayers following adsorption of infectious viruses. RNA was extracted from BGM cells following virus adsorption and washing and then analyzed by a TaqMan assay for enteroviruses. Dr. Di Giovanni found that virus recovery was low for the filtration step (<10%), but high for the host cell capture step (>90%). PCR inhibition presented a problem, with average inhibition of 10% and 85% for 240 ml and 5 L samples respectively, which adversely affected qPCR quantitation. Despite these limitations, he reported reasonable agreement with cell culture assay and 7 of 28 (25%) 240 ml and 2 of 14 (14%) 5 L unseeded samples tested positive for enteroviruses using qPCR. However, additional work is required on sample concentration, inhibition removal, and experimental conditions during cell capture for this promising new method.

Darrell Chandler, Argonne National Laboratory

Dr. Chandler discussed the status of emerging technologies for environmental detection and monitoring of microorganisms that are being developed in his laboratory. He suggested that there are too many pathogens and too many incompatible methods for effective pathogen monitoring. His goal is to develop a simplified and automated flow-through microfluidic process that can detect infectious agents in one liter or less of sample in a few minutes to a couple of hours. His process will use an automated system for

sample concentration and extraction of nucleic acids in conjunction with microarray detectors, which have the ability to detect multiple regions within a gene, multiple genes within a genome, and multiple organisms within a sample. Prototype assays have been developed for *E. coli* O157:H7, *Helicobacter pylori*, and *Cryptosporidium*, among others. Eliminating PCR from the analytical process is a key objective, such that Chandler's group emphasizes the direct detection and analysis of RNA. He described several different microarray adaptations, including "Gel Pad" arrays which had a hierarchical probe design. Hierarchical probes provide internal redundancy and a low false positive/false negative rate. Since there was no amplification of the original genomes, quantitative analysis of multiple genomes may be possible. The next generation of PCR under development was "On-chip PCR", where multiple amplifications, spatial resolution, and detection of products would occur within a single gel pad, giving near real-time results. Dr. Chandler concluded by emphasizing that full automation for routine monitoring is within reach.

Day 1 Summary

Keya Sen, OGWDW, TSC

Dr. Sen summarized the day's presentations and placed them in the perspective of EPA's regulatory needs. Ideally, molecular pathogen detection systems will be developed to provide data for hazard identification, risk assessment, and ultimately, contaminant regulation. Virus methods are composed of two phases: the 'upstream' phase which consists of sampling, elution, concentration and inhibitor removal; and the 'downstream' phase which consists of the PCR assay. The two phases are inseparable and any method development process should consider both phases. Both phases are fraught with difficulties, and the degree of success achieved in each phase will determine the outcome of the assay, most importantly, the sensitivity and specificity of the PCR result. The use of host cell receptors to capture infectious virus particles and subsequent isolation of RNA from these particles for RT-PCR offers promising new technology to capture non-culturable infectious viruses, and also determine the infectivity of those that are culturable. Use of stringent QA/QC policies and procedures, like internal standards, PCR suites, and confirmation of PCR results, etc., are vital.

DAY 2

Ricardo DeLeon, Metropolitan Water District of Southern California

Dr. DeLeon reviewed the elements of standardization necessary to conduct reliable PCR assays and stated that the desired assay end points need to be considered in the standardization process. For example, is it only necessary for the assay to give presence/absence information on viruses, or must the concentration of the viral pathogens and infectivity also be determined? The answers would depend upon whether the PCR was going to be used as a developmental tool or as a monitoring assay. Dr. DeLeon reviewed ground rules and criteria for standardizing PCR primer development and use, PCR reaction conditions, enzyme selection, QC components, and different methods to confirm the identity of PCR products. He opined that PCR is still experimental, rather than standardized science. Few comparative studies have been conducted and published. PCR reactions are optimized locally, and investigators are frequently unable to reproduce another's work. The viruses of interest to EPA are

diverse, for example, adenovirus is a double stranded DNA virus, rotaviruses are double stranded RNA viruses, and echoviruses, coxsackieviruses and noroviruses are single stranded RNA viruses. This diversity in virus type requires unique handling in a PCR assay. Dr. DeLeon believes that available cell cultures are not optimal for many of the viruses sought in water samples, and multiple cell lines are preferable to use of a single cell line. While use of dUTP or UDG, and closed-tube PCR reactions reduce or prevent amplicon contamination, sample preparation and concentration must be separated from analytical activities to minimize cross-contamination of samples. He presented the results of a study of environmental waters and showed that some of the positive findings were due to cross contamination. Among his other QA/QC recommendations were: using performance evaluation samples; using one virus for sample spikes and another virus for cell culture; PCR controls; and sequencing of all positive environmental samples and comparing to sequences of laboratory, spike and cell culture controls. Sequencing needs to be done with reliable methods and following performance criteria that is similar between the sequencing of laboratory strains and environmental samples. Sequencing of laboratory strains or cell culture controls needs to be conducted in triplicate to determine the experimental or sequencing error rate. This is important so that assumptions of rate of change between reference strains and environmental strains of the same virus are not overstated due to sequencing error. He believed that sequencing of the amplicons would help identify the "blind spots" in molecular QA/QC procedures.

Suresh Pillai, Texas A&M University

Dr. Pillai discussed the stability of viruses and viral nucleic acids in the environment. He presented data on the movement of the bacteriophage MS2 through soil and provided evidence that viral RNA molecules from MS2 persist up to three days in groundwater at pH 8.2 and for longer periods of time in waters with a lower pH. He used PCR targeted to four different MS2 genes to show that two of the genes survived for a significantly longer period than the other two. He showed that MS2 RNA binds to sediments and presented data that clay protects the RNA from degradation. Further, preliminary studies suggest that MS2 RNA can be translated into proteins in an *in vitro* system after exposure to groundwater or free chlorine. However, the naked MS2 RNA could infect only those *E. coli* cells that had been specially treated to allow the entry of RNA, thus raising the question of whether viral nucleic acids were infective. He stated that poliovirus RNA in groundwater was degraded faster than MS2 RNA. He concluded by stating that the presence of viral nucleic acids indicates that the distribution system or aquifer has been compromised and suggested that targeting longer nucleic acid fragments for PCR detection would have more chance of predicting intact virus particles.

Dean Cliver, University of California, Davis

Dr. Cliver presented an overview of the structure and function of viruses, emphasizing the difficulty in predicting infectivity based upon PCR assays. He discussed virus inactivation strategies and presented data on thermal, ultraviolet, and sodium hypochlorite inactivation of poliovirus type 1, hepatitis A virus, and feline calicivirus as a surrogate for human noroviruses. Dr. Cliver presented data to show that when intact hepatitis A virus particles were treated with proteinase K and RNase, or either enzyme separately, RT-PCR detected the test viruses. However, after inactivation of virus with hypochloride, as demonstrated by a complete loss of infectivity by plaque assay, RT-PCR assays were negative for virus particles treated with both enzymes and positive when particles are treated with either enzyme separately. He stated that similar results were obtained using inactivation by heating at 72º C or

following inactivation with UV. He showed that thermal inactivation at 37º C inhibited the ability of viruses to attach to host cell receptors, but that inactivated particles were still detected by RT-PCR after treatment with both enzymes. He pointed out that inactivation at low temperatures was what most commonly occurs in the environment and thus further research was needed in this area to find an enzyme combination that would prevent RT-PCR positive reactions on 'dead' viruses. The goals of his research are to find the appropriate enzyme combination, and to extend the studies to other disinfectants as well as viruses that have a food and waterborne route of infection.

Charles Gerba, University of Arizona

Dr. Gerba discussed using data from molecular methods for risk assessment. Quantitative microbial risk assessment models have been validated for *E. coli* O157:H7, *Salmonella*, *Shigella*, *Giardia*, and *Cryptosporidium*. He noted that the end points for federal agencies are different. For example, EPA uses infectivity, while the Food and Drug Administration (FDA) uses infectivity and illness as the indicator of health risk. The dose response relationship is not known for many pathogens of interest to EPA, and the only data available are extrapolated from epidemiological outbreaks or mathematical models. The difficulty in arriving at 'true' infectivity data are illustrated by the fact that many cultured viruses infect at a rate of 1:100 virus particles, while naturally occurring viruses likely infect at a rate of 1:10,000 virus particles, thus experimental results based upon cell culture do not reproduce infectious events in humans. The reliance on cell culture has other drawbacks, because some viruses are not culturable, and other viruses do not produce CPE in cell culture. Attempts to use virus surrogates have not been successful due to seasonal and temporal variation. Dr. Gerba concluded his presentation by citing highlights from Dr. Loge's recent publication on risk assessment using PCR. He stated that the detection limit of PCR assays is dependent upon the recovery of organisms from the sample, the degree of matrix interference manifested as PCR assay inhibition, and the fraction of the concentrated sample subjected to PCR assay. He showed estimates of the minimum volumes of sample that would need to be tested, and shown to be negative, to meet the current EPA guidelines for recreational water.

Frank Loge, Washington State University

Dr. Loge presented a discussion of risk based analysis using PCR data and demonstrated how to extend PCR-based protocols beyond simple detection. A major problem associated with the use of PCR for risk assessment, or in the regulatory context, is how to properly interpret positive and negative results. True positive results will always indicate the presence of contamination, but finding contamination may be unrelated to health risk due to the detection of virus particles that are not infectious. True negative results always need to be interpreted in light of the assay's detection limit. Assays with detection limits too low cannot be interpreted as a lack of potential health risk. Historically, 1:10,000 infections/person/year has been considered an acceptable rate of infection for drinking water exposure, while recreational water exposures of 8:1,000 and 19:1,000 infections/person/year have been established for fresh and marine water, respectively. He showed the maximum infectious pathogen concentrations that would be allowed under this risk standard. For example, rotavirus would meet the standard if it was present at levels up to 0.3 infectious units per 1,000 liters for drinking water, and 1.5-3.4 infectious units per 100 liters for fresh and marine waters. Acceptable detection limits can be calculated from these values. The detection limit is calculated using the volume of sample water filtered, the recovery of virus particles, the fraction of the concentrated sample analyzed by PCR, the sensitivity

of the PCR assay and the dilution factor (that would be needed to remove inhibitory compounds). He calculated the minimum water sample volume that would be needed to achieve acceptable detection limits for a number of pathogens, assuming no inhibition, and 100% recovery. For rotavirus these values were 1.72 liters for marine waters, 3.93 liters for fresh water and 190 liters for drinking water. However, much higher volumes normally will have to be analyzed, because most methods do not give 100% recovery and complete absence of matrix inhibition. Therefore, he emphasized that improvements were needed in sample preparation which included filtration, pathogen recovery and purification. Once these improvements are made, Dr. Loge believes that PCR can be used to determine risk.

Panel Reactions

An invited panel consisting of Dr. Marylynn Yates, University of California, Riverside; Mr. William Yanko, Environmental Microbiology Consultant; and Dr. Jason Jiang, Children's Hospital, Cincinnati, provided insightful comments on the presentations.

Marylynn Yates

Dr. Yates identified three critical decisions that EPA should make before using PCR data for risk assessment. First, EPA must decide whether risk assessment can be performed using detection-only PCR data or whether the PCR data must be associated with evidence of infectivity. Second, she said that EPA should determine whether the standard acceptable levels of risk (e.g., 1:10,000 infections/person/year for drinking water) would be valid when using PCR data in risk assessment. She suggested that the levels might not be valid and that they may differ between viruses. Third, she said that the agency should identify which viruses are of primary interest for making risk assessments. Dr. Yates identified decisions that need to be made to select one or more methods for the viruses of interest that can be standardized and validated. She said that it may not be possible to use the same method for all viruses or for all types of water matrices. She identified questions that need to be asked to evaluate different methods, for example, are quantitative data necessary, or is presence/absence of viruses sufficient, and how will the results be used, since the end use will determine the rigor of the assay? Dr. Yates stated that the Agency should define problem areas where research and development are needed, and support parallel efforts on sample collection and processing, while working to improve detection systems. She also suggested that the Agency identify a minimum set of QA/QC measures that must be incorporated into PCR methods to ensure reliable and reproducible results.

William Yanko

Mr. Yanko emphasized that PCR methods are developmental, especially for environmental samples. He cited comments from a number of presenters, such as "we're not there yet", "we don't know what's going on", and "we don't know what it means". He stated that none of the methods presented are ready for compliance monitoring, since they have not been standardized, validated or correlated with viability/infectivity assays. He believes that PCR is ready for occurrence monitoring, but there is no "best" method. An achievable goal would be to identify the most promising method and validate it for occurrence monitoring studies. He stated that Mark Sobsey provided a good overview of variables, problems and questions to address, while Ricardo DeLeon provided information necessary to construct a decision tree for formatting a particular method. He said that lack of standardization is a major

concern. QC protocols must be developed and standardized prior to use of PCR in occurrence monitoring. The cost of questionable data is unacceptable. Mr. Yanko also pointed out the potential risk of sampling personnel or laboratory workers being a carrier of a virus and unknowingly contaminating the sample. He stated that QA/QC processes must be developed for both upstream and downstream phases of the method. For groundwater and treated water, the method and associated QA/QC must be able to distinguish between low levels of viruses that are randomly distributed in the source water, and low level random laboratory cross contamination. He believes the primary goal should be the development of a consensus PCR protocol, with distinct internal controls and with sufficient reliability to detect a very low level of viruses, for occurrence monitoring of groundwater and treated drinking water.

Jason Jiang

Dr. Jiang reinforced the points made by Mark Sobsey and Kellogg Schwab concerning the importance of QA/QC in production of reliable laboratory results. He urged investigators to seek improved methods for sample volume reduction, or for extracting viruses without concentration, that would result in inhibitor-free volumes suitable for PCR. Dr. Jiang cautioned that many PCR primers are constructed by different laboratories, that are suitable for local surveillance, but may not be useful in occurrence monitoring in other locations. He also cautioned about the increased difficulty in optimizing PCR reactions when multiplex primers are used, stating that typically, the use of multiplex primers reduces the sensitivity of the PCR reaction through competitive inhibition for reagents. He recommended use of limited degenerative primers, differing in one or two bases. He also recommended use of internal controls either as multiplex or in a separate reaction to prevent interference with target DNA or RNA. He next discussed host receptors with respect to caliciviruses and recognition sites on the Lewis secretor and ABO blood group antigens. Secretors are susceptible to norovirus infection while non-secretors are resistant to norovirus infection. Different strain of Norwalk-like viruses recognize different receptors, which may reflect the host range of individual strains. At least four binding patterns have been identified. Methods for detection of Norwalk-like viruses may be developed based on the receptor binding mechanism. He emphasized the importance of basic research in advancement of applied research.

Question and Answer

Panel summary comments were followed by general discussion among the participants in a "Question and Answer" session.

Shay Fout: Are there parts of existing methods that can be incorporated into a unified method?

Marylynn Yates: We can break up the methods and work with the parts of them, and let that drive procedures and processes. We are a long way from regulatory compliance.

Aaron Margolin: Methods will be outdated by the time they can be validated, so a performance-based method approach is needed. Methods must be designed to meet criteria in order to know what the data mean, specifically, do they address the problem? I think it's dangerous to try to carve a method into stone.

Marylynn Yates: EPA should make decisions, determine QA/QC for the parts, evaluate published articles, and interpret the results in light of what you know about the method. We need consensus on what is important.

Suresh Pillai: The disagreement between protocols isn't as broad as some imply. Individual labs are very comfortable with the protocols that they currently use. However, for consensus methods, a lot more still needs to be done to standardize and optimize the protocols. The four lab study that EPA is currently funding is a first step along the way.

Mary Ann Feige: Everyone does their own thing, and it's hard to reconcile the data. Researchers should know what their peers are doing. It's time to standardize parts of the available technology.

Mark Sobsey: We should look beyond fundamentals to what can be done today. There are two main approaches, fishing for nucleic acids or looking for infectious agents. The ICR samples are archived, and they represent a source of study samples of method comparisons. EPA should decide what viruses and samples to study and whether to look for intact viruses, free nucleic acids, or both. EPA must answer the Why? and the Where? before dealing with the How? The ball is in EPA's court. Infectivity is the 'gold standard' for health effects, so culture data are imperative. Unfortunately, some viruses aren't culturable. EPA should articulate an agenda and perspective before gathering all the virologists in the country together. We need to know Why and Where before we're asked How.

David Battigelli: All laboratories don't have the personnel, facilities, or equipment to do 'ideal' work. A realistic approach is needed if environmental laboratories are going to do PCR. What about indirect methods to address public interest? What about using viruses or coliphages as indicators?

Jami Montgomery: We can't always get a single matrix to work. We need flexibility just to comply with the minimum QC standards.

Susan Boutros: Is there a 'strawman' method that will tell the 'consumer' what a positive result means? What data do we need? Are there different methods for different questions?

Mark Sobsey: Labs that can perform cell culture and extract nucleic acids and do hybridizations can skip PCR altogether. Let cell cultures enrich viruses and then use molecular detection by dot blot assays. Reserve PCR for non-culturable viruses and do it only in specialized labs.

Charles Gerba: Characterize source water and establish the appropriate treatment level accordingly. Arizona has PCR machines for bioterrorism and they are becoming more widely available. There's too much obsession with false positive results. False negative results cause more problems.

Frank Loge: What defines EPA's water programs? This question must be answered before addressing how PCR technologies can be integrated into water quality criteria. Infectivity is most important in finished water, but is it really that important in source water?

Mary Ann Feige: EPA's Office of Ground Water and Drinking Water needs occurrence data in finished water.

Mark Sobsey: Yes, and for recreational water monitoring, stakeholders want a 2-hour test! We have MCL's for chemicals, and there's a need for commercial kits for PCR.

Susan Boutros: My laboratory would like to request non-pathogen control strains so they don't have to deal with etiologic agents.

Shay Fout: Standardized controls are a first step.

Keya Sen: Armored RNA technology has produced synthetic viral particles that serve as internal controls for detecting HIV and HCV by PCR. They are being used by the transfusion blood industry, such as the American Red Cross, for monitoring the presence of these viruses in blood. Perhaps we could use the technology to produce internal controls for waterborne viruses.

?????: Market pressure is moving utilities to consider reuse of water so that wastewater becomes a source water for drinking water treatment.

Mary Ann Feige: Do sampling and recovery show a difference between large volume and multiple small volumes? How do we spike large volumes of water?

Mark Sobsey: MS-2 is being used for phage spiking for UV disinfection studies and phage is used as a surrogate for training techs to extract and do PCR.

Summary

George Hallberg thanked the participants for a lively discussion and concluded the meeting with the following summary:

EPA will hold a meeting to review the results of the four laboratory study, and it is hoped that some of the questions posed at this meeting will become clear at that time. Among the next steps, EPA and the research community should consider what QA/QC procedures exist in clinical and forensic laboratories using PCR, and incorporate those that are appropriate to environmental samples. A limitation to using performance-based methods is the lack of a benchmark or gold standard. Other questions that must be addressed include selection of standards, controls and primers to ensure reliability of PCR results. EPA should decide how to deal with infectivity determination if PCR results are to be used in risk assessment. Question such as, "Is groundwater at risk?", or "Is there human health risk?" or "Is the disinfection process efficient?" will determine whether infectivity data are required. EPA desires a core method with modular add-ons according to special circumstances.

Open Discussion

Mark Sobsey: We are not the only industry using PCR. Forensics, food, paternity, etc., must be correct, and have stringent protocols, although their samples are easier to deal with. We can't jump to regulation before validation. Performance based methods must still have a baseline. What is the baseline? That question leads right back to the decision making tree.

Dean Cliver: The ultimate goal of PCR methods is to assess infectivity. The first steps are the same, not two separate methods, just two options of the same method.

Frank Loge: We have made rapid advances in our ability to detect pathogens using PCR. However, our ability to characterize infectivity using PCR is grossly underdeveloped, and is many years from being a developed and scientifically defensible technology. Is assessing infectivity necessary in characterizing risk? Absolutely. But in the short term, as we develop PCR assays to assess infectivity, I would contend that we should take the next logical step forward and develop risk-based standards using conventional PCR assays that quantify the concentration of targeted organisms, independent of infectivity. This approach would be most appropriate to implement in source waters, not finished waters, and would provide a conservative characterization (e.g., not all organisms detected would be viable) of water quality. As PCR assays are developed to characterize infectivity, this framework can be adapted to finished waters.

Gerard Stelma: A PCR method must address infectivity. Failing to do so can lead to unfounded public panic.

Conclusion

The goals of the January 2003 workshop were to evaluate existing PCR QC protocols for monitoring viruses in water, to identify techniques for improving the reliability of PCR detection of viruses in an effort to develop a standard QC protocol that could be properly validated, to identify further areas of research for molecular detection of viruses, and to assess the feasibility of PCR-based methods for use in risk assessment activities.

PCR and other nucleic acid based molecular detection methods offer the promise of replacing cell culture and immunoassays for detection of viruses in environmental samples. While PCR-based methods for detection of viruses have been advancing rapidly in recent years, the currently available methods and their associated QA/QC procedures have not been standardized, sampling strategies and sample concentration methods do not facilitate adequate virus recovery, and the sensitivity of PCR assays has not proven to be adequate for monitoring viruses in environmental samples. Direct PCR methods by themselves do not differentiate between infective and non-infective viruses, which is necessary for risk assessment activities. Method complexity and variability has thwarted development of standardized protocols and QA/QC procedures, and no validated method is currently available for virus detection. Workshop experts recommended continued collaboration toward developing a standardized

QC protocol that can be properly validated for monitoring and regulatory applications. Internal quality assurance controls and sequencing of PCR products are needed to assure reliability of assay results.

As a result of the workshop, EPA is developing a draft QA/QC manual for nucleic acid based protocols as a first step toward development of standardized PCR protocols that can be properly validated. Researchers will be encouraged to adopt these new QA/QC procedures and to actively collaborate to bring some degree of standardization to a PCR protocol that can be used for virus monitoring.

Originally, EPA envisioned another molecular methods workshop to be held in June 2003, however, the agency has postponed that meeting to provide additional time for researchers to deal with sample and protocol issues raised at the January 2003 workshop.