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Chapter 13

Recent Advances and Future Needs in Environmental Virology

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The global water quality inventory and environmental virology: status and implications

Medically important viruses were first noted as part of the environmental “malaises” early in human history (often described with symptoms such as jaundice) but it was advances in cell culture, electron microscopy, and immunology that spurred the discovery and characterization of human viruses. The first isolations of viruses from water came in the 1950s and 1960s for surface waters and drinking waters, respectively (Gerba and Rose, 1989). Yet it had long been understood that enteric viruses such as poliovirus were shed in feces and thus by association present in sewage and sewage-polluted waters. Our conventional definition of environmental virology has primarily focused on enteric viruses and contaminated drinking water, fecal-oral transmission, and associated person-to-person transmission. Likewise, the management and control of waterborne viruses has focused on disinfection of drinking water and vaccinations. Despite the tremendous improvements in water and sanitation management fueled by a better understanding of the nature of viruses, emerging viruses such as the polyomaviruses and reemerging epidemics of age-old viruses such as poliovirus, as well as concerns associated with intentional use of eradicated viruses such as the smallpox virus, challenge our conventional definition of “environmental virology” and traditional approaches to control.
The global outbreaks of severe acute respiratory syndrome (SARS) and avian influenza (AI) highlight the degree of vulnerability that high-density urban populations face when threatened by novel, unanticipated viral pathogens. This is further underscored by security fears brought on by recent acts of terrorism both in the US and abroad. Less sensational but equally serious outbreaks of many other viruses like norovirus, hantavirus, and West Nile virus have been documented worldwide and are on the rise. Rotavirus-induced diarrhea is still the most prevalent infant killer in many developing nations causing an estimated 140 million cases worldwide and killing almost 600,000 people annually (Parashar et al., 2003).

A United Nations report on the world water crisis situation has highlighted the twin issues of scarcity and impacted quality of the world’s drinking water supply (UNESCO, 2003). Scarcity of water in many parts of the world has forced people to turn to increasingly less pristine sources of water for their drinking and other needs. This has given rise to an increased incidence of waterborne disease. The burgeoning world population is also placing a greater strain on the current world water supply. Irrigation currently consumes approximately 70–80% of the world’s fresh and groundwater supply while polluting lakes and streams through surface runoff of pesticides, fertilizers, and land-applied biosolids with their associated pathogen loads. The US Food and Agricultural Organization (FAO) anticipates a net expansion of irrigated land of some 45 million hectares in 93 developing countries to a total of 242 million hectares in 2030 and projects that agricultural-water withdrawals will increase by some 14% from 2000 to 2030 to meet future food production needs (FAO, 2002).

The increase in population also means that more wastewater is being generated. Globally, 41% of the population is without adequate sanitation and very little of the global wastewater is treated. (WHO/WSH main site: www.who.int/water_sanitation_health/). According to the US House Transportation Subcommittee on Water Resources (2003), after investing $250 billion in wastewater infrastructure in the US with the passage of the Clean Water Act of 1972, our communities’ economic well-being, which relies on clean water, and our ability to continue to meet the public health goals are at risk. This is despite there being 16,000 publicly owned wastewater treatment plants, 100,000 major pumping stations, 600,000 miles of sanitary sewers, and 200,000 miles of storm sewers in the US.

Historically, the importance of protecting one’s drinking water supply has been well documented and recognized. Poisoning or contaminating an enemies’ water supply has been practiced in warfare since at least the fourth century BC. Less deliberate acts of contamination occur more frequently due to industrial accidents, inclement weather, and weak enforcement of regulations or operator neglect. While animal wastes have been implicated in bacterial and parasitic outbreaks, the viruses remain associated with some of the most serious health consequences such as the outbreak of viral hepatitis E in Kanpur, India in 1991 that affected an estimated 79,091 people (Naik et al., 1992) with 30% mortality in pregnant women in the first trimester. The recent widespread poliovirus outbreaks throughout Africa are likely...
in part due to contaminated water and the inadequate and wastewater treatment (Pavlov et al., 2005).

More recently, attention has also focused on the need to protect recreational water sources (Wade et al., 2003; Standish-Lee and Loboschefsky, 2006). Fresh and salt water sources represent an important recreational resource, especially to economies that rely heavily on tourism. In addition, the increasing scarcity of pristine water sources has meant that the water cycle is being short circuited in order to provide adequate water for drinking, recreation, power generation, agriculture, and industrial processing. The assessment of the impairment of waterways for the various uses based on the “indicator bacteria” and *Escherichia coli* has not provided enough specificity in regard to health risk, sources of the pollution, identification of the responsible party, and control. This has fueled a demand for advanced pathogen detection.

In the twenty-first century, viral diseases have changed the landscape of medicine. Acquired immunodeficiency syndrome (AIDS) now infects millions of people worldwide and up to 30% of the populations in Africa. Waterborne diseases will be particularly devastating to these individuals and the list of potential waterborne viral agents is growing. Certain microbiological advances like the polymerase chain reaction (PCR) and microarray technology may provide the tools necessary for monitoring any new agent of interest. The era of pathogen discovery is not over. We should be prepared to use all the microbiological advances to continue to understand the transmission of diseases through water. This must then be coupled with our knowledge of engineering to determine how to maintain and develop the quantity and quality of our water resources. With the advances made in medicine, microbiology, and engineering we can add to the existing body of knowledge and continue to advance the field of “environmental virology”.

**Advanced pathogen detection and discovery**

The detection of viruses in water and other environmental samples constitutes special challenges. The standard method of detection of viral pathogens in environmental samples uses assays in mammalian cell culture. The infected cell cultures undergo observable morphological changes called cytopathogenic effects (CPEs) that are used for the detection of viruses. Even though many viruses are culturable in several cell lines and are thus detectable by the development of CPEs in cell culture, there are several viruses, like enteric waterborne adenoviruses types 40 and 41, which are difficult to culture and do not produce clear and consistent CPE. Other viruses, like waterborne caliciviruses, have not yet been successfully grown in cell cultures. Conventional cell culture assays for the detection of viruses in environmental samples have limited sensitivity and can be labor-intensive and time-consuming. Two advances, the PCR and microarrays, have spurred the study of viruses and should be further applied to the field of environmental virology.
PCR application for the study of environmental virology

Since only a small percentage of viruses are cultivatable, there is a need to examine in a more systematic fashion the development of standard/consensus methods for the collection and processing of viruses in the water environment via molecular techniques. Table 1 lists some of the critical issues in the application of PCR techniques in environmental virology.

The PCR-detection assay allows for highly sensitive and highly specific detection of virus nucleic acid sequences. PCR was initially used as a research tool for the amplification of nucleic acid products. It is fast gaining acceptance in the clinical diagnostic setting and it has been effectively applied to the detection of viruses from environmental samples. The sensitivity of PCR has been demonstrated to be comparable or superior to cell culture (Raboni et al., 2003; Lee and Jeong, 2004). In order to use PCR one must already know the exact sequence of a given region of the viral nucleic acid. Primers are then designed to amplify that specific region of the genome. Primers for the specific detection of many of the enteric viruses have been published. Nested and multiplex PCR, which are variations of the conventional PCR, have also been applied to virus detection in water samples. The chief drawback of PCR methods is that they are incapable of distinguishing between active and inactive targets.

The latest advancement in molecular methods is the development of quantitative real-time PCR. Quantitative PCR (qPCR) or real-time PCR can be used to quantify the original template concentration in the sample. Following DNA extraction, real-time PCR simultaneously amplifies, detects, and quantifies viral acid in a single tube within a short time. In addition to being quantitative, real-time PCR is also faster than conventional PCR. Real-time PCR requires the use of primers similar to those used in conventional PCR. It also requires oligonucleotide probes labeled with fluorescent dyes, or an alternative fluorescent detection chemistry different from conventional gel electrophoresis, and a thermocycler that can measure fluorescence. For quantification, generation of a standard curve is required from an absolute standard with known quantities of the target nucleic acid or organism. When real-time PCR quantitative results for adenoviruses in environmental samples were compared with conventional cell culture results, it was concluded that the real-time PCR method demonstrated higher quantities of adenoviruses in comparison with conventional techniques. This was likely due to the presence of noninfectious viruses and low sensitivities of tissue culture assays to serotypes present in the environmental samples (Choi et al., 2004; He and Jiang, 2004).

Another modification of the standard PCR method, integrated cell culture (ICC)-PCR makes use of a cell culture step to enhance sensitivity and demonstrate infectivity. The ability to detect only infectious viruses among many noninfectious viruses is important for predictions of public health risk in water and other environmental samples, particularly after disinfection. As described above, molecular methods detect part of the viral DNA or RNA that indicates virus presence but not infectivity. To determine infectivity, molecular methods can be used in conjunction
### Table 1

**Critical issues for PCR applications to environmental virology**

| Category                                      | Details                                                                                                                                                                                                                                                                                                                                 |
|-----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample concentration and inhibition control  | Environmental samples are less concentrated than clinical samples. Sample pre-concentration, usually by filtration and elution of large volumes of water, is required prior to DNA extraction. PCR in environmental samples is often inhibited by various substances. Sample dilutions and sample pre-treatment methods should be evaluated for inhibition control. |
| Molecular techniques and quantification      | For quantification, the use of real-time PCR is required along with generation of a standard curve from an absolute standard with known quantities of the target nucleic acid or organism. Real-time PCR requires the use of primers similar to those used in conventional PCR. It also requires oligonucleotide probes labeled with fluorescent dyes and a thermocycler that can measure fluorescence. |
| Molecular techniques and infectivity determination | Instead of CPEs, detection of bacterial mRNA by reverse transcription (RT)-PCR assay can be used to detect infectivity. In the case of DNA viruses, detection of viral mRNA during cultivation is an indication of the presence of infectious viruses. In the case of RNA viruses detection of the double-stranded replicative form in cell cultures inoculated with viruses demonstrates infectivity (Cromeans et al., 2005). |
| Specificity and primer and probe design      | PCR and real-time PCR primers should be designed to amplify only the DNA or RNA of interest. Real-time PCR primers differ from conventional PCR primers. The primer selection in the case of real-time PCR is restricted due to requirements of smaller target amplicon sizes than the PCR target sizes. Also, in the case of real-time PCR there is a need to select probes that meet certain criteria. |
| QA/QC requirements                           | The high sensitivity of the molecular techniques requires demanding quality assurance, quality control protocols. PCR techniques are very sensitive to contamination from amplified DNA. Contamination can result in false negatives and false positives. Appropriate lab infrastructure and highly trained personnel are required for effective QA/QC (EPA, 2004). To avoid false-positive results, confirmation of amplification products, such as sequencing is required. |
with cell cultures. The ability of viruses to infect cell cultures and to replicate their nucleic acid implies that they are also capable of infecting the human host.

Targeting specific messenger RNA (mRNA), after inoculation of cell cultures with samples containing infectious viruses, cultures are observed for evidence of virus replication. Instead of CPEs, detection of viral mRNA by reverse transcription (RT)-PCR assay can sometimes be used to detect infectivity. DNA viruses, such as waterborne adenoviruses, form mRNA during replication in cells. Only infectious adenoviruses can enter cells and transcribe mRNA during replication; inactivated virus will be unable to do so. Therefore, detection of viral mRNA during cultivation is indicative of the presence of infectious viruses. Positive-strand RNA viruses, such as waterborne enteroviruses and noroviruses, produce a negative, complementary strand of RNA in the early phase of viral replication. This negative strand of RNA is bound to newly formed positive strands of RNA to form a double-stranded replicative form; detection of the replicative form in cell cultures inoculated with viruses can also be used to demonstrate infectivity (Cromeans et al., 2005).

A PCR case study for environmental virology

Human adenoviruses are considered as emerging waterborne viruses. They are currently listed in the Environmental Protection Agency’s Contaminate Candidate List. The importance of adenoviruses and the potential health risks associated with their waterborne transmission has been recognized by the scientific community (Fong and Lipp, 2005). Currently, there are 51 different types of human adenoviruses. Adenoviruses are a common cause of gastroenteritis, upper and lower respiratory system infections, and conjunctivitis (Swenson et al., 2003). Other diseases associated with adenoviruses include acute and chronic appendicitis, cystitis, exanthematous disease, and nervous system diseases (Swenson et al., 2003). Adenoviruses are considered important opportunistic pathogens in immunocompromised patients (Wadell, 1984).

The potential of transmission of adenoviruses through water is suggested by the findings of several researchers. Enriquez et al. (1995) concluded that enteric adenoviruses are more stable in tap water and wastewater than poliovirus. Irving and Smith (1981) reported that adenoviruses are more likely to survive conventional sewage treatment than enteroviruses. In addition, Hurst et al. (1988) estimated that most adenoviruses detected in wastewater may be enteric adenoviruses. Borchardt et al. (2003) associated diarrhea of viral etiology (including adenoviruses) with drinking municipal water. Adenovirus outbreaks have also been associated with recreational exposure and swimming (Foy et al., 1968; Cardwell et al., 1974; D’Angelo et al., 1979; Martone et al., 1980; Turner et al., 1987; Papapetropoulou and Vantarakis, 1998; Harley et al., 2001).

Adenoviruses have been found in wastewater samples in significant numbers in different geographic locations (Irving and Smith, 1981; Krikelis et al., 1985a, b; Hurst et al., 1988, Puig et al., 1994; Greening et al., 2002; He and Jiang 2005). Girones et al. (1995) presented data that showed the prevalence of adenoviruses in
sewage samples. The presence of adenovirus has also been reported in river waters (Tani et al., 1995; Cho et al., 2000; Greening et al., 2002; Choi et al., 2004; Lee et al., 2004; Choi and Jiang, 2005; Haramoto et al., 2005; Van Heerden et al., 2005) and a river estuary (Castignolles et al., 1998). Also, Jiang et al. (2001) detected human adenoviruses in urban runoff-impacted coastal waters in southern California, and Fong and Lipp (2004) detected adenoviruses in the coastal reaches of a river in Georgia.

Adenoviruses occurrence has been reported in drinking waters. Chapron et al. (2000) detected adenovirus in untreated surface waters collected and evaluated by the information collection rule. Fourteen of the 29 samples (48%) were positive for adenoviruses and 38% of these samples were determined to be infectious. Van Heerden et al. (2003) reported incidence of adenoviruses in raw and treated drinking water in South Africa. The results indicated human adenoviruses present in (13%) of the raw and (4%) of the treated water samples tested. At a later study, Van Heerden et al. (2005) reported adenovirus in 10 of 188 drinking water samples. Cho et al. (2000) and Lee and Kim (2002) detected adenoviruses in tap water.

To control the problem of adenovirus infections there is a need to further evaluate environmental exposure pathways and assess human exposure risk. Developing reliable and sensitive detection methods is crucial to this effort. It is also critical that the methods be able to quantify and differentiate between serotypes. Advances in molecular techniques have been applied in the detection of adenoviruses. Table 2 presents a summary of molecular techniques that have been applied to this complex group of viruses. Many of these assays have been used for detection in water. There is no approved, universally applied method for the detection of adenoviruses. Further methods development, comparison of existing techniques and evaluation of sensitivity and specificity of techniques is required to advance the state of knowledge relative to human adenoviruses and waterborne infections.

Microarrays and other hybridization-based detection

Microarrays were first described in 1995 by Schena et al. (1995). They are arrays of spots on specially prepared glass or silicon surfaces. Each spot on an array serves as a single test at which a hybridization of DNA, immunological attachment, or chemical reaction can occur. These arrays can be useful for screening multiple samples against multiple targets but they are neither as sensitive nor as specific as PCR or cell culture. Microarray technology is enabled by the ability to deliver submicroliter volumes of material to an attachment surface or matrix. DNA microarrays are arrays in which DNA–DNA/DNA–cDNA or DNA–RNA hybridization reactions are an indication of a positive/negative reaction. Spotted DNA microarrays consist of either PCR generated or synthesized oligonucleotides that have been printed or mechanically spotted on to specially coated glass slides. In situ synthesized arrays have their probes chemically synthesized directly on to the support matrix.
Table 2
Summary of molecular methods applied to the detection of human adenoviruses

| Species* | Serotype | Target gene | Primer name | Sequence 5'-3' | Length | Amplicon size | Wastewater & water samples | Type of assay | Source |
|----------|----------|-------------|-------------|----------------|--------|--------------|-----------------------------|---------------|--------|
| A–F      | All 51   | Hexon       | JTVXF       | GGA-CGC-CTC-GGA-GTA-CCT-GAG | 21     | 96           | No                          | Real-time PCR | Jothikumar et al., 2005 |
|          |          |             | JTVXR       | ACI-GTG-GGG-TTT-CTG-AAC-TTG-TT | 23     |               |                             |               |        |
| A–F      | 1–8,11   | Hexon       | NA          | GAC-ATG-CTC-GAT-CCT-GAG-GTC-GAT-CCC-ATG-GA | 29     | 140          | No                          | Real-time PCR | Watanabe et al., 2005 |
| A–F      | All 51   | Hexon       | NA          | C(AG)C-GGG-CA(ACT)-GCA-CCA-G | 19     | 68           | No                          | Real-time PCR | Formiga-Cruz et al., 2002 |
| A–F      | All 51   | Hexon       | AD1         | CTG ATG TAC TAC AAC AGC ACT GGC AAC ATG GG | 32     | NA           | No                          | PCR           | Sarantis et al., 2004 |
| A–F      |          |             | AD2         | GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT | 38     |               |                             |               |        |
| A–F      | 1–5, 9, 16, 17, 19, 21, 28, 37, 40, 41, 25 | Hexon       | AD2         | CCC-TGG-TAK-CCR-ATR-TTG-TA | 20     | NA           | Yes                         | Real-time PCR | He and Jiang, 2005; Choi and Jiang, 2005 |
| A–F      | All 51   | Hexon       | AD3         | GAC-TCG-TCW-GTS-AGY-GGG-C | 19     |               |                             |               |        |
| A–F      | All 51   | Hexon       | Hex1        | TTC-CCC-ATG-GCI-CA-ATG-GAC | 20     | 482          | Yes                         | Nested PCR    | Ko et al., 2003; Xu et al., 2000 |
| A–F      |          |             | Hex2        | CCC-TGG-TAG(CT)-CC(AG)-ATAM-TTG-TA | 20     |               |                             | Multiplex PCR  |        |
| A–F      | All 51   | Hexon       | AQ1         | GCC-AGC-GTG-GGG-TTT-CTA-AAC-TC | 23     | 129          | Yes                         | Real-time PCR | Heim et al., 2003; Haramoto et al., 2005; van Heerden et al., 2005 |
| A–E      | 2,3,4,7,11,21,11 | E1A       | AdE1A-F     | GCC-TGC-AGC-ATC-TGT-ATG-AT | 20     | 409–446      | No                          | Multiplex PCR  | Lin et al., 2004 |
| A–F      |          |             | AdE1A-R     | TCT-GAT-ATA-GCA-AAG-CGC-ACA | 21     |               |                             |               |        |
| A–E      | 2,3,4,5,7,16,21 | Fiber     | AdFib-F3    | ACT-GTA-KCW-GYT-TTG-GYT-GT | 20     | 430–437      | No                          | Multiplex PCR  | Lin et al., 2004 |
| A–E      |          |             | AdFib-R3    | TTA-TTS-YTG-GGC-WAT-GTA-KGA | 21     |               |                             |               |        |
| Column | Row | Type | Primer Name | Sequence | Length | Method   | Reference                      |
|--------|-----|------|-------------|----------|--------|----------|--------------------------------|
| A–E    | 3,4,6,7,16,21 | Hexon | AdHex-F7 | CAC-GAY-GTG-ACC-ACM-GAC-CG TTK-GGT-CTG-TTW-GGC-ATK-GCY-TG | 20 | 770-815 | No Multiplex PCR | Lin et al., 2004 |
|        |      |      | AdHex-R5 | GGA-CGC-CTC-GGA-GTA-CCT-GA CGC-TGI-GAC-CIG-TCT-GTG-G | 23 |        |                          |                      |
| C,D,F  | (1,2,5,6),(8,19),(40,41) | Hexon | JHKXF | GGA-CGC-CTC-GGA-GTA-CCT-GA CGC-TGI-GAC-CIG-TCT-GTG-G | 20 | 135 | No Real-time PCR | Ko et al., 2005 |
|        |      |      | JHKXR | TTK-GGT-CTG-TTW-GGC-ATK-GCY-TG | 19 |        |                          |                      |
| A      | All | Fiber | NA | TGC ATT TAG TGT TTG ATG AA ATA GGT TTA GAT GTA TCT CCC TGT AA | 20 | NA | No Multiplex PCR | Pehler-Harrington et al., 2004 |
| A      | 31  | Hexon | 31F | AGA-TAT-GAC-ATT-TGA-AGT-TGA-CCT-GTAC-GCT-CGT-CCA | 25 | NA | No Multiplex PCR | Gu et al., 2003 |
| A      | Fiber | AdA1 | GCT-GAA-GAA-MCW-GAA-GAA-AAT-GA | 23 | 1444–1537 | No Multiplex PCR | Xu et al., 2000 |
|        |      |      | AdA2 | CRT-TTG-GTC-TAG-GGT-TTG-CAC-GAAT-CGC | 21 |        |                          |                      |
| B      | All | Fiber | NA | TCT TCC CAA CTC TGG TAC CCT GGG TTT ATA AAG GGG TG | 18 | NA | No Multiplex PCR | Pehler-Harrington et al., 2004 |
| B      | Fiber | AdB1 | TST-ACC-CYT-ATG-AAG-ATG-AAA-GC GGA-TAA-GCT-GTA-GTR-CTG-GGC-AT | 23 | 670–772 | No Multiplex PCR | Xu et al., 2000; Lin et al., 2004 |
| B      | Fiber | AdB2 | GCT-GTA-ATG-GTA-ATG-AAA-GC GGA-TAA-GCT-GTA-GTR-CTG-GGC-AT | 23 |        |                          |                      |
| C      | All | Fiber | NA | TCA TAT CAT GGG TAA CAG ACA T CCC ATG TAG GCG TGG ACT TC | 22 | NA | No Multiplex PCR | Pehler-Harrington et al., 2004 |
| C      | 2,5 | E1A | AdC-E1AF | CCA-CCT-ACC-CTT-CA-ACT-GAA-CG CTG-GTC-GCA-GGT-AAG-ATC-G | 20 | 260 | Yes PCR | Ko et al., 2003 |
|        | 2,5 | AdC-E1AR | CCA-CCT-ACC-CTT-CA-ACT-GAA-CG CTG-GTC-GCA-GGT-AAG-ATC-G | 19 |        |                          |                      |
| C      | All | Fiber | AdC1 | TAT-TCA-GCA-TCA-CTT-CCT-TTC-TT | 22 | 1988–2000 | No Multiplex PCR | Xu et al., 2000 |
|        |      |      | AdC2 | AAG-CTA-TGT-GGT-GGT-ATG-GGG-GC | 20 |        |                          |                      |
| D      | All | Fiber | NA | CTC GGG GTG GAA GAT GAC T ATT GGG TCA GCC AGT TTG AGT | 19 | NA | No Multiplex PCR | Pehler-Harrington et al., 2004 |
| Species | Serotype | Target gene | Primer name | Sequence 5'-3' | Length | Amplicon size | Wastewater & water samples | Type of assay | Source |
|---------|----------|-------------|-------------|----------------|--------|---------------|-----------------------------|---------------|--------|
| D       | All      | Fiber       | AdD1        | GAT-GTC-AAA-TTC-CTG-GTC-CAC | 21     | 1205-1221     | No                          | Multiplex PCR | Xu et al., 2000 |
|         |          |             | AdD2        | TAC-CCG-TGC-TGG-TGT-AAA-AAT-C | 22     |               |                |               |        |
| E       | All      | Fiber       | NA          | TTG GCT CAG GTT TAG GAC TCA GT CTG TTA AGG GTA ATC TTT ATA TTC CCT TCC-CTA-CGA-TGC-AGA-CAA-CG | 23     | NA            | No                          | Multiplex PCR | Pehler-Harrington et al., 2004 |
|         |          |             | AdE1        | TCC-CTA-CGA-TGC-AGA-CAA-CG | 20     | 967           | No                          | Multiplex PCR | Xu et al., 2000 |
|         |          |             | AdE2        | AGT-GCC-ATC-TAT-GCT-ATC-TCC-ATG-TAT-TCC-TTC-GCA-AAC-TTC-CA | 21     |               |                |               |        |
| F       | NA       | Hexon       | Adenovirus.est.fwd | ATG-TAT-TCC-TTT-TTC-GCA-AAC-TTC-CA | 23     | 244           | No                          | Real-time PCR | Logan et al., 2006 |
|         |          |             | Adenovirus.est.rev | GCC-ACA-TGG-TGC-GAT-GGC-A | 19     |               |                |               |        |
| F       | 40,41    | Fiber       | JTVFF       | AAC-TTT-TCT-TCT-TAA-TAG-ACG-CC | 23     | 118           | No                          | Real-time PCR | Jothikumar et al., 2005; Ko et al., 2005 |
|         |          |             | JTVFR       | AGG-GGG-CTA-GAA-AAC-AAA-A | 19     |               |                |               |        |
| F       | 40       | Hexon       | f-AD157     | ACC-CAC-GAT-GTA-ACC-ACA-GAC-A | 22     | 88            | Yes                         | Real-time PCR | Jiang et al., 2005 |
|         |          |             | r-AD245     | ACT-TTG-TAA-GAG-TAG-GCG-GTT-TCC | 24     |               |                |               |        |
| F       | 40,41    | Fiber       | NA          | AAC ATG CTC ATC CAA ATC TCG CCT A TTC AGT TAT GTA GCA AAA TAC AGC | 25     | NA            | No                          | Multiplex PCR | Pehler-Harrington et al., 2004 |
|         |          |             |             | AAA TAC AGC | 24     |               |                |               |        |
| F       | 40,41    | E1B         | 4041-1      | CTG-ATG-GAG-TTT-TGG-AGT-G | 19     | NA            | No                          | Multiplex PCR | Rohayem et al., 2004 |
|         |          |             | 4041-2      | CCA-TTA-GCC-TGC-TCC-TTA | 18     |               |                |               |        |
| F       | 40,41    | E1A         | AdF-E1AF    | GGG-AAC-TGG-GAT-GAC-GT | 17     | 280           | Yes                         | PCR           | Ko et al., 2003 |
|         |          |             | AdF-E1AR    | CCS-TCT-TCA-TAG-CAT-TTG | 18     |               |                |               |        |
| F       | 40,41    | Fiber       | AdF1        | ACT-TAA-TGC-TGA-CAC-GGG-CAC-TAA-TGT-TTG-TGT-TAC-TCC-GCT-C | 21     | 541–586       | No                          | Multiplex PCR | Xu et al., 2000 |
|         |          |             | AdF2        |                   |        |               |                |               |        |
| F     | 40,41 | Hexon | hexAA1885  | GCC-GCA-GTG-GTC-TTA-CAT-GCA-CAT-C  | 25 | 308 | Yes  | PCR, Nested PCR, Multiplex PCR  | Allard et al., 1990; Puig et al., 1994; Girones et al., 1995; Pina et al., 1998; Castignolles et al., 1998; Cho et al., 2000; Chapron et al., 2000; Greening et al., 2002; Maluquer de Motes et al., 2004; Lee et al., 2004; Rigotto et al., 2005; van Heerden et al., 2005; Choo and Kim, 2006 |
|-------|-------|-------|------------|-----------------------------------|----|-----|------|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
|      |       |       | hexAA1913  | CAG-CAC-GCC-GCG-GAT-GTC-AAA-GT     | 23 |      |      |                                 |                                                                                                                                                    |
| F     | 41    | E1B   | 41AA142    | TCT-GAT-GGA-GTT-TTG-GAG-TGG-CTA   | 24 | 2187| No   | PCR                             |                                                                                                                                                    |
|       |       |       | 41AA358    | AGA-AGC-ATT-AGC-GGG-AGG-GTT-AAG   | 24 |      |      |                                 |                                                                                                                                                    |
| F     | 40,41 | E1A   | 40AA45     | ATT-GCT-GTT-GGC-GCT-TTT-GAC-ATA-G | 25 | 858 | No   | PCR                             | Allard et al., 1990                                                                                                                                  |
|       |       |       | 41AA129    | TCA-AGA-GGA-CTT-GGG-GCG-CTT-TAA   | 24 |      |      |                                 |                                                                                                                                                     |

*Note: A–F species include all 51 human infectious species.*
Microarrays have conventionally been used to develop gene expression profiles of certain targets of interest. Increasingly, research has also focused on adapting microarray technology to screen clinical specimens against multiple target pathogens in a highly efficient manner (Zhou, 2003; Bodrossy and Sessitsch, 2004). Microarrays have been designed for the detection and genotyping of hepatitis B virus, adenoviruses, Epstein-Barr virus, herpes simplex virus, influenza virus, and human papillomavirus (Sengupta et al., 2003; Boriskin et al., 2004; Korimbocus et al., 2005; Min et al., 2006; Song et al., 2006).

Proposals have been put forth for using DNA microarrays as an environmental detection tool and possible biodefense tool (Pannucci et al., 2004; Sergeev et al., 2004). Only a few examples exist for the application of microarray technology on environmental samples. For example, Kelly et al. (2005) have used DNA microarrays to analyze the nitrifying bacterial community in a wastewater treatment plant. Wu et al. (2004) developed a community genome array that was able to reveal species and strain differences in microbial community composition in soil, river, and marine sediments.

The use of microarrays as an environmental research tool can be divided into two broad categories: arrays that serve to detect specific gene sequences regardless of source and arrays that target specific pathogens. Straub and Chandler (2003) have proposed that a unified system for the detection of waterborne pathogens would significantly advance public health and microbiological water analysis and have indicated that advances in sample collection, on-line sample processing and purification, and DNA microarray technologies may form the basis of a universal method to detect known and emerging waterborne pathogens. Table 3 summarizes some of the viral microarrays and their applications.

A number of commercially available microarray chip platforms are currently available. Their main differences are the manufacturing method employed and feature density. Affymetrix GeneChip arrays are able to accommodate up to 1.3 million unique features on a 5” × 5” quartz wafer and are manufactured using a photolithographic masking technique (Pease et al., 1994). Agilent microarrays have a 44,000 feature set and are synthesized using an inkjet printing method (Hughes et al., 2001). Febit’s Geniom microarrays contain only 6000 features but hybridization can be carried out with eight chips in parallel allowing multiple sample processing (Guimil et al., 2003). Nimblegen microarrays contain 3,90,000 probes per array and are manufactured using a micromirror focusing technique. Less expensive glass slide arrays for smaller probe sets are also within the in-house fabrication capability of most research institutions (approximately 16,000 features per slide). Current limitations of the technology include issues regarding validation and low starting microbial biomass (Wu et al., 2006) and need to be addressed before the technology may be applied.

A microarray case study: community-level monitoring for viruses

At present, the monitoring of public health occurs at the individual patient level. The highly disseminated nature of the public health system, however, means that it
| Virus types                                      | Numbers of gene sequences | Application                                   | Major finding                                                                                     | References                |
|------------------------------------------------|---------------------------|----------------------------------------------|---------------------------------------------------------------------------------------------------|---------------------------|
| Influenza A virus                               | 12,000 features           | Subtyping and sequencing                     | Integrated microfluidic system. Mismatch discrimination is achieved at the enzymatic ligation step. | Liu et al. (2006)         |
| Varicella-zoster virus (VZV)                    | 5 pairs of oligonucleotide probes 18–21 mer long 8 probes | Distinguish 3 major circulating genotypes of VZV | Evaluated against 6 reference strains and 130 clinical specimens                                   | Sergeev et al. (2006)    |
| Animal pestiviruses DNA suspension microarray   | Approximately 22,000 oligonucleotide probes | Detection and differentiation of animal pestiviruses | 40 strains of CSFV, BVDV1, BVDV2 and BDV tested                                                  | Deregt et al. (2006)     |
| Pan-viral DNA microarray, virochip ver. 3       | 110 oligonucleotide probes | Detected human parainfluenza virus 4 (HPIV-4) | Conventional clinical laboratory testing using an extensive panel of microbiological tests failed to yield a diagnosis. Microarray worked. | Chiu et al. (2006)       |
| Six species of orthopoxvirus (OPV)              |                           | Simultaneous detection and identification of six species of OPV including Variola, Monkeypox, Cowpox, Camelpox, Vaccinia, and Ectromelia viruses. | The method allowed us to discriminate OPV species from VZV, herpes simplex 1 virus (HSV-1), and HSV-2 that cause infections with clinical manifestations similar to OPV infections. | Ryabinin et al. (2006)   |
| Respiratory pathogen                            | No data                   | 20 common respiratory and 6 category A biothreat | The results demonstrate a novel, timely, and unbiased method for                                    | Wang et al. (2006)       |
| Virus types                        | Numbers of gene sequences | Application                                      | Major finding                                                                                                                                                                                                 | References                  |
|-----------------------------------|---------------------------|--------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| microarray ver 1 (RPM v.1)        |                            | pathogens known to cause febrile respiratory illness | the molecular epidemiologic surveillance of influenza viruses. Identification of a Novel Gammaretrovirus in Prostate Tumors, and strongly implicate RNase L activity in the prevention or clearance of infection \textit{in vivo}. | Urisman et al. (2006)       |
| Pan-viral DNA microarray, virochips | 1592 probes, 25-mer       | Virus discovery and identification                |                                                                                                 |                             |
| Four major serotypes of dengue virus |                            | Detection and identification                      | Host-blind probe design                                                                                                                                  | Putonti et al. (2006)       |
| Flu-chip 55 diagnostic microarray  | 216 probes 22-mer         | Detection and subtyping of influenza A, B and Avian influenza H5N1 | The combined results for two assays provided the absolutely correct types and subtypes for an average of 72% of the isolates, the correct type and partially correct subtype information for 13% of the isolates, the correct type only for 10% of the isolates, false-negative signals for 4% of the isolates, and false-positive signals for 1% of the isolates. | Townsend et al. (2006)      |
| Epstein-Barr virus genome-chip     | 71 PCR amplified fragments, 12 control DNA fragments | Detects gene expression patterns of EBV in tumor cells | This study demonstrates that the EBV-chip is useful for screening infection with EBV in tumors, which may lead to insights into tumorigenesis associated with this virus.                                  | Li et al. (2006)            |
| Universal viral chip | No data | Characterization of all currently known viruses in Genbank | Have designed virus probes that are used not only to identify known viruses but also for discerning the genera of emerging or uncharacterized ones. | Chou et al. (2006) |
|----------------------|---------|----------------------------------------------------------|---------------------------------------------------------------------------------|------------------|
| Enterovirus microarray | 13 probes | Detecting and differentiating EV71 and CA16 | 144 clinical specimens examined. Diagnostic accuracy of 92.0% for EV71 and 95.8% for CA16. Diagnostic accuracy for other enteroviruses (non-EV71 or -CA16) was 92.0%. | Chen et al. (2006) |
| Affymetrix resequencing RPM v.1 VZV expression microarray | No data | Species- and strain-level identification of respiratory viruses Displays gene expression profile of VZV | Was able to show differences in levels of transcriptions among the various VZV ORFs 23 different FMDV strains representing all seven serotypes were detected and typed by the FMD DNA chip. | Lin et al. (2006); Kennedy et al. (2005) |
| Foot-and-mouth disease (FMD) DNA chip | 155 probes, 35–45 mer long | Detection and typing of FMDV serotypes and differentiation from other viruses causing vesicular diseases | | Baxi et al. (2005) |
| CNS viral pathogen chip | 40,588 probes 20-mer | Identify herpes simplex virus type 1 (HSV-1), HSV-2, and cytomegalovirus; all serotypes of human enteroviruses and five flaviviruses (West Nile virus, dengue viruses and Langat virus) | Able to detect the 3 major CNS disease-causing viruses from a single sample | Korimbocus et al. (2005) |
| Virus types                          | Numbers of gene sequences | Application                                                                 | Major finding                                                                                                         | References       |
|-------------------------------------|---------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|------------------|
| Flavivirus microarray               | 8 probes, 500 nucleotides long | Detect and distinguish between yellow fever (YF), West Nile, Japanese encephalitis (JE), and the dengue 1–4 viruses | Verified on all 7 flavivirus types. Detects and identifies even diverged strains of West Nile and Dengue virus | Nordstrom et al. (2005) |
| Ligation-detection microarray       | 6 detection sites          | Detection and genotyping of SARS coronavirus (SARS-CoV)                      | 20 samples assayed with the universal microarray were confirmed by DNA sequencing                                       | Long et al. (2004) |
| Hepatitis B and D virus chip        | 14 probe fragments         | Hepatitis D and Hepatitis B virus detection                                 |                                                                                                                       | Zhaohui et al. (2004) |
| Pan viral CNS chip                  | 38 gene targets for 13 viral causes of meningitis | Detects and differentiates between echoviruses, HSV-1 and 2, VZV, human herpesvirus 7, human herpesvirus 6A and 6B, Epstein-Barr virus, polyomavirus JC and BK, cytomegalovirus, mumps and measles viruses |                                                                                                                       | Boriskin et al. (2004) |
takes either a long time or a massive influx of cases before a disease outbreak is recognized. The trend towards increasingly urbanized and dense city living and the more frequent travel between communities necessitates that community health monitoring adopts a more proactive preventative role instead of merely recording and reporting disease data. In order to do so, there need to be tools that are able to screen for the large panel of possible viral pathogens which are representative of the pathogen loads present in the larger community. To meet this requirement it becomes logical to monitor the community’s sewage using microarrays designed to detect the presence of waterborne pathogens.

Using the OligoArray version 2.1 software written by Rouillard et al. (2003), Wong et al. (2006) have designed a total of 780 probes to detect 25 of the common virus families known to cause gastroenteritis. Two additional probes were placed unto the microarray as quality control sequences for fabrication. The 25 virus families targeted in this microarray are hepatitis A and E virus, human adenovirus A–F, noroviruses, sapoviruses, human enterovirus groups A–E, polioviruses, rotavirus groups A–C, coronaviruses, astroviruses, human cytomegalovirus, torovirus, polyomaviruses, and picobirnaviruses. These oligonucleotide probes were synthesized unto a 63 × 116 microarray by the Gulari Research Group at the University of Michigan (Gao et al., 2001).

Figure 1 illustrates the processing steps required to prepare a sample for hybridization on an array. Environmental samples are first concentrated using conventional virus concentration methods like organic flocculation, tangential flow filtration, or ultrafiltration. Concentrates are then biologically amplified by passage through cell culture and virus nucleic acid is extracted. Labeling of viral nucleic acid is

![Fig. 1 The processing steps required to prepare a sample for hybridization on an array.](image-url)
carried out through the use of DNA polymerase or RT enzymes. The labeled nucleic acid can then be hybridized on the microarray and a distinctive hybridization pattern is produced when target viruses bind to their complementary probes on the array.

Testing of the virus microarray was carried out using poliovirus virus LSC-1 and adenovirus type 40 and 41 as test subjects. Poliovirus was cultured on Buffalo green monkey (BGM) cells and adenoviruses were cultured on MA104 cells and their respective nucleic acid contents were extracted. Poliovirus RNA was labeled using the Superscript Indirect Labelling System (Invitrogen, Inc. Carlsbad, CA). Adenovirus DNA was labeled using the large fragment of DNA polymerase I (Klenow Fragment). Hybridization was carried out using an in-house hybridization wash station at 20°C for approximately 17 h. Subsequently, the microarray was scanned using a Molecular Devices model 4000B Genepix Scanner (Molecular Devices Corporation, Sunnyvale, CA), washed at 25°C, scanned, and subsequently washed and rescanned at 1°C intervals to generate a washing curve profile. Figure 2 illustrates the hybridization profile obtained from the hybridization of poliovirus, adenovirus type 40 and 41 with the virus microarray. Excellent signal to noise was achieved and the viruses were easily identified and distinguished.

Current work demonstrates that the chip can be used with viral concentrates from cell culture systems originating from sewage, with unique virus detection in community wastewater indicative of infections in the population. Thus future applications suggest, as with PCR, it will be a combination of conventional methods used in environmental virology and new techniques that will enhance pathogen discovery.

**Prediction of the future: trends and needs in environmental virology**

Viruses remain a public health concern and should remain a priority for the water and health community. These bio-nano particles are excreted in high concentrations
by infected individuals, have high potency (probability of infection is high with low numbers (Haas et al., 1999)), and are environmentally robust. The ability of both DNA viruses and RNA viruses to rapidly evolve means new and emerging viral pathogens will need to be addressed. Pathogen discovery and characterization, occurrence in the environment, exposure pathways, and health outcomes via environmental exposure need to be addressed. This will likely follow a new microbial risk framework, which will require focused research on some important properties of viral disease transmission. The future will require models that examine community risks and provide explicit links between the models currently under development for environmental exposure and infectious disease.

It is predicted that there will be in each of these areas some key scientific issues that will need to be addressed (Table 4). New advances like qPCR and microarray technology should be applied to wastewater streams for viral pathogen discovery and for characterization of disease in our populations. These data should be used to further an analysis of water quality and health status. Adenoviruses, polyomaviruses, noroviruses, and other respiratory viruses need to be characterized, as does respiratory viral transmission particular via recreational exposure. Greater assessment of

Table 4
Predicted needs for advancing the field of environmental virology

| Needs for method development in environmental virology | Advances |
|--------------------------------------------------------|----------|
| Quantification                                         | Real-time PCR |
| Infectivity                                             | Cell-culture PCR, mRNA detection |
| Specificity                                             | Primer sequence databases |
| Quick response time                                     | Real-time PCR, biosensors |
| Multiple detection                                      | Microarrays, multiplex PCR |

| Areas of interest in environmental virology             |
|---------------------------------------------------------|
| Area                                                    | Focused interest |
| Virus discovery and characterization                     | Community assessment |
|                                                        | Cancer viruses |
|                                                        | Obesity viruses |
|                                                        | Heart viruses |
|                                                        | Continued global spread of poliovirus |
|                                                        | Respiratory viruses |
|                                                        | Animal viruses |
|                                                        | Viruses in sewage |
|                                                        | Recreational waters |
|                                                        | Storm waters |
|                                                        | Rural waters (ground, septic, agricultural waters) |
|                                                        | Irrigation waters |
| Human exposure pathways and risk assessment              | |


the occurrence of animal viruses in water, their role in animal health, and application to source tracking are needed. Finally, environmental virology needs to include the detection, occurrence, and transmission of viruses from fomites. The health impacts of these viral pathogens in nontraditional outcomes such as cancer are future areas requiring research.

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