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EXAMINING THE PRESENCE AND PREVALENCE OF KEY HUMAN ENTERIC VIRUSES IN ENVIRONMENTAL SAMPLES USING CULTIVATION, MOLECULAR AND ARRAY-BASED TOOLS FOR DETECTION

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By

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Abstract

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The current recreational water standards are based on the measurement of fecal indicator bacteria and do not always provide an accurate indication of viral pollution. Unfortunately, enteric viruses are difficult to detect using conventional cell culture methods as it requires several days to complete, making it unsuitable as a routine monitoring tool. Molecular detection methods like integrated cell culture polymerase chain reaction (PCR) have been successfully used to rapidly detect the presence of virus nucleic acid from environmental samples but with over a 100 different types of viruses capable of causing infections in humans, routine testing using PCR is not feasible. In this dissertation, I describe the development of a virus microarray that can be integrated with cell culture to rapidly perform high throughput screening of environmental samples.

The microarray holds a total of 780 unique probes broadly targeting 27 different groups of enteric viruses known or suspected to cause enteric disease and was used to test the hypothesis that the range of virus types present in the environment is under-estimated by conventional cell culture and cell culture-PCR. To test this hypothesis, the types of viruses present at two typical recreational beaches in the Great Lakes were characterized using conventional methods to serve as a baseline for the types of viruses present in the environment. In addition, raw sewage samples which are the major contributing source of human enteric viruses present in the environment, were analyzed using both PCR

methods and the virus microarray over a period of thirteen months. Using PCR, 12 species of viruses from 7 major viral groups were detected as opposed to 14 different groups of viruses detected using microarrays. A detailed analysis of the results showed the following: 16 cases in which both the PCR and the microarray results were positive and in agreement. 17 cases in which the microarray was positive but could not be supported by PCR results, 15 cases in which PCR results were positive for an enteric viruses but were not shown to hybridize significantly on the microarray and 303 instances in which neither PCR nor the microarray produced a positive signal. This resulted in a p value of less than 0.0001 for a Fisher's exact test indicating strong statistical correlation between the PCR and the microarray. It was observed that while PCR was more sensitive, able to detect viruses during months where no virus signals were detected on the microarray, the group specific primers used often biased the results in favor of just one species of virus when the array was able to indicate the presence of several members from the same family or related families of viruses.

We conclude that microarrays are capable of screening for a broad number of pathogenic viruses which may be circulating in the population and excreted in the community wastewater but that PCR remained a more sensitive detection method. This is the first demonstration of an environmental microarray for detection of viruses in water and could be used to improve public health surveillance.

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ABBREVIATIONS

CL – confidence limit

DNA – deoxyribonucleic acid

dNTP – deoxy-nucleotide triphosphate

L – liters

M – molars

mM – millimolar

MPN – Most Probable Number estimate

PCR – polymerase chain reaction

PFU – plaque forming units

RNA – ribonucleic acid

CHAPTER 1 INTRODUCTION TO HUMAN VIRUSES AND THE WATER ENVIRONMENT

1.1 Viral Gastroenteritis, Disease Surveillance and Outbreak Information

Viruses which cause gastroenteritis, which is an inflammation in the gastrointestinal tract, are known as enteric viruses and are found in contaminating sewage, surface and groundwater. They are primarily associated with drinking recreational and food-borne disease though some enteric viruses are capable of causing more severe diseases like meningitis and cardiomyopathy. In 2002, the World Health Organization (WHO) estimated that there were 1.7 million deaths related to unsafe water, sanitation and hygiene, mainly through infectious diarrhea (WHO, 2002). The estimated 4 billion cases of diarrhea annually account for over 82 million disability adjusted life years (DALYs) and represents 5.7 percent of the global morbidity (Pruss and Havelaar, 2001). In the United States, there were 764 documented waterborne outbreaks attributed to drinking water between 1971 and 2002, resulting in 575,457 cases of illness and 79 deaths (Blackburn et al., 2004). The most recent report from the US CDC reported that within the 2003-2004 time period, there were 98 waterborne disease outbreaks for both drinking and recreational waterborne outbreaks (5458 illness, 58 hospitalization and 5 deaths) , 50 of which were caused by infectious agents leading to gastroenteritis. Viruses were known or suspected to have been responsible for 8 out of 50 of the infectious gastroenteritis cases reported (Dziuban et al., 2006; Liang et al., 2006).

These outbreak figures however do not represent the true numbers of waterborne disease, the possible etiological agents and the cost. An estimate of the waterborne infection and illness rate in the US by Reynolds et al postulated that 10.7 million infections per year and 5.4 million illnesses per year occur in populations served by community groundwater systems; 2.2 million infections per year and 1.1 million illnesses per year occur in non-community groundwater systems; and 26.0 million infections per year and 13.0 million illnesses per year occur in municipal surface water systems. The total estimated number of waterborne illnesses per year in the U.S. was thus estimated to be 19.5 million per year (Reynolds et al., 2008).

1.2 Productivity Losses, Economic Costs of Viral Infections

While the symptoms of the diarrhea are generally mild in adults and the majority of sufferers recover within a few days, the annual productivity loss due to rotavirus infections alone has been estimated to be in excess of 6.3 million pounds in the United Kingdom and 352 million dollars in the United States (Barnes et al., 1998). Within the state of Michigan, tourism, fishing, boating and other recreational activities involving contact with water contributes over 17.5 billion dollars to the State's economy and generates over 192,700 jobs. In 2006, 4 percent of the 207 regularly monitored tier 1 beaches in Michigan were found to exceed the State's daily maximum bacterial standard of 300 colony forming units per 100 milliliters of water. Nationwide, the number of closings and advisory days at Ocean, bays, and Great Lakes beaches for 2006 increased by 28 percent compared to the previous year resulting in a total of 25,643

closing/advisory days. The majority of these closings (over 67 percent) were in response to known pollution events or actual monitoring results (Dorfman and Stoner, 2007).

1.3 Knowledge Gaps, Limitations of Current Tests

Despite the large economic and productivity costs attributed to enteric viral disease. Limited data are available on the prevalence of viruses in the environment. Previous work on surveying the environment for the presence of enteric viruses have been restricted by the cost and manpower needed to analyze such matrices. Although bacterial phages have been proposed as an alternative indicator of viral pollution, research has shown that they do not adequately represent the extent and degree of human and animal viral pollution in the environment (Jiang and Chu, 2004).

In 2000, Congress authorized the "Beaches Environmental Assessment and Coastal Health Act of 2000" which required that the US Environmental Protection Agency develop within 5 years "new or revised water quality criteria for pathogen and pathogen indicators (including a revised list of testing methods, as appropriate)". To date, no official testing methods for the detection of pathogenic viruses in the environment have been forthcoming. This is despite the fact that it is generally accepted that the health impact of waterborne diseases due to viruses is greatly underestimated (Leclerc et al., 2002). Most of the bacterial and protozoal causes of gastroenteritis are well characterized and detection is generally possible within 24-48 hours. Viruses are harder and more laborious to detect but yet are believed to survive longer in the environment and also have a higher probability of causing an infection even at low numbers. Being

obligate intracellular parasites, they have to be cultured in animal cell lines in order to demonstrate infectivity. Some viruses however, are refractory to cell culture and can only be detected via molecular methods like reverse transcription PCR or via methods like electron microscopy or enzyme immuno assays, which are costly and limited to a small number of central laboratories.

It is currently estimated that there are approximately over 100 individual species of viruses and an undetermined number of sub-strains of viruses that are capable of causing infections in humans through drinking and recreational use. These viruses are excreted in the feces and urine of infected individuals and may survive exposure to the environment long enough to infect other individuals. In Michigan and the Great Lakes, while drinking water quality is generally satisfactory, there is growing concern about the fresh water coastal beaches, especially due to sewage inputs. It is hypothesized that sewage represents a significant source of viral pathogens and that the detection of viruses represents a sewage pollution risk to bathers at recreation sites. Hundreds of viruses have been reported in sewage, yet no approach has been made available to screen widely for the presence of these viruses in environmental matrices.

1.4 Microarray Detection of Pathogens

Within the past 10 years, microarray technology has been increasingly adapted for use as a multiple pathogen screen tool. Initially used as a means of performing gene expression analysis, microarrays now exist for the detection of a diverse list of pathogens from parasites, to bacteria and viruses. The use of microarrays in an environmental

setting is still in its infancy however and a microarray designed to perform high throughput analysis of water samples for the presence of waterborne disease-causing viruses would represent a novel application of this technology.

CHAPTER 2. LITERATURE REVIEW

2.1 Recreational Water Quality Standards

Recreational water quality for safe swimming is regulated at the state level under the *Clean Water Act,* as are the quality of ambient waters and sewage discharges. From a microbiological perspective, public health protection is the goal and fecal indicator bacteria have been used to develop water quality standards and criteria. For recreational water use, states have been encouraged to adopt standards that are at least as stringent as the recommendations set by the EPA under Section 304 of the Clean Water Act (EPA, 2003) and the Beaches Environmental Assessment and Coastal Health (BEACH) act. The current EPA guideline for safe swimming and full body contact is 126 colony forming units (cfu) / 100ml for *E. coli* and for enterococci the level is 33 cfu / 100ml and 35 cfu / 100ml for freshwater and marine waters respectively. The state of Michigan has adopted a recreational water quality standard for monthly averages of no more than 130 cfu / 100 ml for surface waters protected for full body contact with no daily sample average (3 samples per site) more than 300 cfu/100 ml and a single sample maximum of no more than 1,000 cfu / 100 ml for surface waters protected for partial body contact.

In the state of Michigan, the National Pollution Discharge Elimination System that regulates sewage discharges uses a 30-day geometric mean standard of 200 fecal coliform bacteria per 100 milliliters and a 7 day geometric mean standard of 400 fecal coliform bacteria per 100 milliliters for treated and untreated sewage discharges. This standard was originally set up to protect "swimmable" waters where sewage discharges

impacted recreational sites and generally list segments of waterbodies under an impairment which would require assessment. It has been recognized however that the presence of pathogenic viruses is not well correlated with the numbers of indicator bacteria and that the standards for indicator bacteria used to protect recreational waters are disconnected from the rules that govern sewage discharges and public safety. Thus characterization of pathogenic viruses in particular may be of particular importance in these types of waters if in the future better assessment of disease potential from swimming in sewage polluted waters is to be undertaken.

2.2. The Enteric Viruses

Viruses were first identified by Russian biologist Dmitry Ivanosky in 1892 and initially referred to as filterable agents. They are very small microorganisms straddling the border of the biological definition of life. Their sizes range from 20nm to 400nm and they lack many of the characteristics of a biological cell – they have no cell wall, no internal membranes and no cellular machinery to reproduce with. They are thus obligate intracellular parasites, relying upon host machinery in order to multiply. Viruses historically were classified according to their host specificity, shape and size as seen under the electron microscope. As more and more viruses were discovered, and more tools for their characterization were developed, classification began to include their nature, nucleic acid, and the presence or absence of an envelope. The classification system developed by David Baltimore lists 7 categories of viruses (Group I to VII) which includes viruses capable of infecting humans, animals, plants, fungi and bacteria

(Baltimore, 1971). A table is provided below showing the seven categories as well as a few examples from each (Table 2.1).

The International Committee on Taxonomy of Virus (ICTV) has established a virus classification scheme based upon a set of 2600 character criteria. The new Linnean-like classification adopts a virus taxonomy of order, family, subfamily, genus, and species and viruses are classified based on the relatedness of genome sequence, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physiochemical properties of virions, and antigenic properties of viral proteins (Buchen-Osmond, 2003). It also allows subspecies, serotype and strain/isolate classification of viruses. Currently there are close to 2000 virus species in the latest report from ICTV (Fauquet et al., 2005).

It has been estimated that almost half of all emerging pathogens arising from infections caused by contact with water are caused by viruses or small infectious protein particles called prions (Taylor et al., 2001). Viruses are reported to be the main acute diarrhea outbreaks in infants and young children worldwide (Kapikian, 1996). Mead *et al.* have estimated that 80% of the 38.6 million annual cases of gastroenteritis in the United States are the result of a viral infection (1999). Enteric viruses are viruses spread by the fecal-oral route as they cause infection through replication in the gastrointestinal system and are subsequently excreted in feces. They are found thus in sewage and are the main groups associated with contamination of food and water. The major families of enteric viruses can roughly be grouped into the following families - the *Picornaviridae*,

the *Adenoviridae*, and the *Reoviridae*. More recently, the noroviruses and other caliciviruses in the family *Caliciviridae* have gained prominence for being the causative agent of several recent outbreaks (Bohnker and Thornton, 2003; Doyle et al., 2004; Drinka, 2005; Falkenhorst et al., 2005; Fretz et al., 2005; Korsager et al., 2005; Maunula et al., 2005; Maunula and Von Bonsdorff, 2005; Sakon et al., 2005; Seto et al., 2005; Hjertqvist et al., 2006; Ike et al., 2006; Koopmans et al., 2006; Takkinen, 2006; Vainio and Myrmel, 2006; Vidal et al., 2006; Ljubin-Sternak et al., 2007; Tu et al., 2007; Fukuda et al., 2008; Makary et al., 2008; Torner et al., 2008).

Table 2.1. Baltimore Classification of viruses by genome type

DNA viruses	
Group I – dsDNA viruses (double stranded DNA)	Enterobacteria phage T4 Human herpesviruses Cowpox virus, Adenoviruses, Enterobacteria phage λ, Polyomavirus
Group II – ssDNA viruses (single stranded DNA)	Parvovirus B19
RNA viruses	
Group III – dsRNA viruses (double stranded RNA)	Rotavirus
Group IV – (+) ssRNA viruses (positive single stranded RNA or mRNA like)	Coronavirus Norwalk Virus Hepatitis E virus West Nile Virus Hepatitis C virus Poliovirus Rhinovirus Hepatitis A virus
Group V – (-)ssRNA viruses (negative single-stranded RNA)	Influenza viruses
DNA and RNA Reverse Transcribing viruses	
Group VI – ssRNA-RT viruses (single stranded RNA)	HIV1
Group VII – dsDNA-RT viruses (double stranded DNA)	Hepatitis B virus

2.2.1 The Enteroviruses

Enteroviruses are small, single stranded, positive sense RNA viruses. They are non-enveloped and have an average diameter of 27-30 nanometers. Enteroviruses are a large group of viruses from the *Picornaviridae* family that are responsible for many infections in children. These viruses live in the intestinal tract, but can cause a wide variety of illnesses. There are sixty-six distinct serotypes of enterovirus known to cause infection in humans: three polioviruses, twenty-three Coxsackie A viruses, six Coxsackie B viruses, twenty-eight echoviruses and six other enteroviruses (Bruu, 2003). Enteroviruses can cause respiratory infections, gastrointestinal infections, skin infections and neurological infections.

Large numbers of enteroviruses are routinely found in sewage but few waterborne outbreaks due to this group of viruses have been reported (Metcalf et al., 1995). One of the suggested reasons is that multiple symptoms are exhibited by enteroviral infections and thus the lack of a common unifying symptom results in failure to recognize an outbreak by the medical community. While the entry of polio, coxsackie, echo, or other enteroviruses through the gut may cause incidental mild diarrheal symptoms, it is the spread of the virus through the bloodstream to other organs (e.g., central nervous system, heart, pleura, pancreatic islets) that produces major disease manifestations - examples of which are hand-foot-and-mouth (HFM) disease, herpangina, myocarditis, neonatal sepsis and pleurodynia. Research has also indicated that there is a link between recent enteroviral type 71 infections and type 1 diabetes (Elfaitouri et al., 2007; van der Werf et al., 2007; Frisk et al., 2008; Oikarinen et al., 2008).

2.2.2 The Adenoviruses

Adenoviruses are classified as non enveloped double stranded DNA viruses. Their average diameter is between 60-90 nanometers. They are medium sized viruses and have a genome of approximately 30 kilobasepairs. There are 5 groups of human adenoviruses with more than 40 subtypes. Adenoviruses are widely recognized causes of respiratory, ocular, and genitourinary infections. However, serotypes 40 and 41 (previously called fastidious enteric adenoviruses) primarily affect the gut, contributing to 5%-20% of hospitalizations for childhood diarrhea in developed and developing countries (Giordano et al., 2001; Rodriguez-Baez et al., 2002; Filho et al., 2007; Sdiri-Loulizi et al., 2008). Adenovirus is considered to be only second to rotaviruses in causing acute viral gastroenteritis worldwide. The peak incidence is among children less than 2 years of age, but older children and adults may be infected, with or without symptoms. Infections occur throughout the year with no clear peaks (Wong et al., 2008). Other serotypes of adenovirus, particularly type 2, 7, 12 and 31, have also been associated with diarrhea (Noel et al., 1994; Harsi et al., 1995; Li et al., 2005). Adenoviruses have frequently been detected in immuno-compromised patients with diarrhea. A study examining 377 HIV-positive patients presenting with diarrhea found that adenoviruses were present in 7.2% of them and accounted for 50% of the patients who were enteric-virus positive (Thomas et al., 1999). However other studies have made it unclear whether the presence of adenoviruses in HIV-positive enteric virus-infected patients was associated with the presentation of diarrhea (Liste et al., 2000).

2.2.3 The Rotaviruses

The rotaviruses are double stranded RNA viruses from the family *Reoviridae* with a genome consists of 11 segments. Rotaviruses are the most common cause of severe diarrhea among children. In the United States, approximately 3.5 million cases occur each year. A review of the national rates, trends, and risk factors for diarrhea- and rotavirus-associated hospitalizations and deaths among children <5 years of age by Fischer et al. (2007) found that rotavirus infections remain the most important cause of pediatric diarrhea throughout the study period of 1993-2003, causing approximately 60,000 hospitalizations and 37 deaths annually. Worldwide, an estimated 140 million cases occur each year, causing almost 600,000 deaths (Parashar et al., 2003).

In the United States, the peak incidence of rotavirus diarrhea is among children 6 months-2 years of age, although in developing countries younger infants may be affected. By 4 years of age, most persons have been infected and are immune to this severe dehydrating syndrome, but a high inoculum or lowered immunity can still produce milder illness among older children or adults, among travelers to developing nations, the elderly, and persons with debilitative or immunosuppressive conditions. A person with rotavirus diarrhea may excrete approximately 1×10^{12} infectious particles/milliliter of stool (Iturriza-Gomara et al., 1999). Asymptomatic rotavirus excretion has been reported among half of children the day before diarrhea starts and among one-third during the week after symptoms end (Pickering et al., 1988). Many children can shed rotavirus and never become ill (Champsaur et al., 1984; Champsaur et al., 1984). In a small prospective study in the UK, rotavirus caused 4·1% of acute diarrhea in adults admitted to hospital

(Jewkes et al., 1981). Similarly, 3% of acute diarrhea in Switzerland (Loosli et al., 1985), 3% of infectious diarrhoa pathogens in a Swedish clinic for infectious diseases (Svenungsson et al., 2000), 5% of adults with gastroenteritis requiring admission in Thailand (Echeverria et al., 1983), 2–4% of adults older than 15 years with gastroenteritis presenting to their family physician in the Netherlands (de Wit et al., 2001), and nearly 4% of individuals older than 45 years in Michigan were due to rotavirus (Koopman and Monto, 1989). Even higher rates of infection have been seen. In Japan, Nakajima *et al.* (2001) reported that group A rotavirus had a role in 14% of patients with diarrhea. Pryor *et al.* (1987) noted that rotavirus was second only to *Campylobacter spp* as a cause of diarrhea among Australian adults, accounting for 17% of all cases. In Indonesia, 42% of patients presenting with diarrhea had rotavirus-positive stools compared with 11% of control samples (Oyofo et al., 2002). In a study of Mexican adults, 63% of patients presenting with acute gastroenteritis during winter months were positive for rotavirus (del Refugio Gonzalez-Losa et al., 2001).

Nosocomial rotavirus among pediatric populations is common; in one study among hospitalized children, a nosocomial infection rate of 1.6 per 1000 child-days was recorded (Snelling et al., 2007). Rotavirus at day-care centers, in both endemic and outbreak form, is also common (Gabbay et al., 1999; Floret et al., 2006). Outbreaks in neonatal units are frequently reported, but infection among full-term infants is usually benign, perhaps because maternal antibody transferred during the third trimester protects against illness for the first 3-6 months of life (Bishop et al., 1996; Nguyen et al., 2006); premature infants are at higher risk. Among adults, only one outbreak arising from

rotavirus contamination of a municipal water supply has been reported (Gallay et al., 2006).

It was previously thought that only Group A rotaviruses were capable of causing an infection in humans while the other antigenic groups (B-E) were zoonotic. In 1982, however, an epidemic of Group B rotavirus affected millions of persons in China (including adults, children, and neonates) (Hung et al., 1984), and since then outbreaks have re-ocurred, although affecting fewer persons. Studies of immunoglobulin pools from Shanghai suggest that the Chinese population had been exposed to this pathogen in the past (Penaranda et al., 1989).

2.2.4 The Noroviruses

Noroviruses are single, positive-stranded RNA viruses from the family *Caliciviridae*. They are only 27 nanometers in diameter which makes them one of the smallest among the viruses. A British study suggested that approximately 3% of children hospitalized for diarrhea excrete calicivirus (Ellis et al., 1984), and a U.S. study found approximately the same percentage (2.9%) for children with diarrhea in day-care centers (Matson et al., 1989). On the basis of antibody-prevalence studies of pooled immunoglobulin and serum samples from many parts of the world, most persons appear to have been infected by age 12 (Sakuma et al., 1981; Parker et al., 1994). Norovirus has been reported to be highly seasonal and geographic, with peaks in the winter for the northern hemisphere and peaks in the late spring and summer for the southern hemisphere (Marshall et al., 2003), though summertime peaks in the northern hemisphere have been documented (Lopman et al., 2003).

Person-to-person transmission is presumed to be essential for endemic disease, but for enteric viruses, contaminated shellfish, cold foods, and drinking water have been implicated as vehicles (Wanke and Guerrant, 1987; Stolle and Sperner, 1997). A survey of foodborne disease outbreaks in Australia between 1995 and 2000 identified six outbreaks caused by noroviruses or 3% of the 214 documented outbreaks for that period (Dalton et al., 2004). Most outbreaks of norovirus gastroenteritis were observed to be community based, affecting nursing homes, schools, cruise ships, camps, restaurants, and military installations (CDC, 2002; CDC, 2003). A study of 233 US outbreaks occurring between July 1997 and June 2000 reported that the most common occurrences of outbreaks were in restaurants and catered meals (39%) and in nursing homes (25%). Contaminated food was the most common vehicle of transmission (57%) followed by person-to-person contact (16%) and contaminated water (3%) (Fankhauser et al., 2002).

2.2.5 The Astroviruses

Astroviruses are very similar to noroviruses in that they are small positive stranded RNA viruses. Astroviruses are from the family *Astroviridae* and are divided into eight human species (HAstV-1 to HAstV-8). Their genome length is between 6800 and 7900 nucleotides. Initially, astroviruses were regarded as only a minor contributor to the incidence of childhood diarrhea based on Electro Microscopy (EM) results. More recent serological testing has shown astroviruses to be more prevalent than previously thought. Currently, astroviruses are considered second only to rotaviruses as the cause of gastroenteritis in infants and young children. Studies of hospitalized children suggest that

astroviruses may account for 4-10% of admissions for diarrhea (Palombo and Bishop, 1996; Guerrero et al., 1998; Bon et al., 1999; Pang and Vesikari, 1999; Rodriguez-Baez et al., 2002). Other studies have indicated that the prevalence might be higher among children evaluated for gastroenteritis in the ambulatory setting (Cruz et al., 1992).

A long term study in Australia over four consecutive years showed that the pattern of astrovirus infection that was without a statistically significant seasonal peak (Mustafa et al., 2000). Other studies however have described a predilection of astrovirus for winter or the rainy season in populations living also in temperate regions (Cruz et al., 1992; Maldonado et al., 1998). A study by Michell et al. found that the seroprevalence of 393 infants and children to HAstV-1 decreased from 67% in infants <3 months of age to 7% by 6 to 8 months of age. Antibodies to HAstV-3 exhibited a lower prevalence, with 26% positive at <3 months, and 0% at 6 to 11 months. This was consistent with loss of transplacental antibodies. Children acquired HAstV-1 antibody with a peak prevalence of 94% at 6 to 9 years of age and HAstV-3 antibodies with a peak prevalence of 42% by 6 to 9 years of age (1999).

2.2.6 The Picobirnaviruses

Picobirnaviruses are bi-segmented, non-enveloped, double stranded RNA viruses that are as yet unclassified. Prior to a study done by Pereira and colleagues, picobirnaviruses were only thought to infect animals. Pereira and colleagues identified picobirnaviruses using polyacrylamide gel electrophoresis among fecal specimens from children with diarrhea in Brazil (Pereira et al., 1993). Picobirnaviruses have also been

detected in stool samples from HIV positive individuals both with and without diarrhea (Grohmann et al., 1993; Gonzalez et al., 1998; Giordano et al., 1999). Though still primarily associated with young children, the elderly and immunocompromised individuals, a survey of fecal specimens collected between 1982 and 1993 from the United Kingdom detected picobirnaviruses in patients with and without gastroenteritis and throughout the age range of 3 to more than 65 years (Gallimore et al., 1995).

2.2.7 The Parvoviruses

Parvoviruses are the smallest of all human viruses, measuring 20-25 nanometers in diameter. They are single-stranded DNA viruses with a genome size between 3 to 5 kilobasepairs. Only one gastrointestinal parvovirus outbreak has been documented among humans (Christopher et al., 1978) though several outbreaks have occurred among animals (Palmer and Thornley, 2004). The relationship of these particles to disease is unclear, but they have been associated with shellfish-related outbreaks of gastroenteritis (Appleton, 1987).

Human parvovirus B19 is another member of the *Paroviridae* family that causes pathogenesis in human but unlike gastrointestinal parvovirus, human parvovirus B19 is transmitted via the respiratory route. Human parvovirus B19 causes erythema infectiosum and, particularly in adults, acute symmetric polyarthropathy. In the immunocompromised host persistent B19 infection is manifested as pure red cell aplasia and chronic anemia. Likewise, the immature immune response of the fetus may render it

susceptible to infection, leading to fetal death in utero, hydrops fetalis, or development of congenital anemia (Heegaard and Brown, 2002).

2.2.8 The Toroviruses

Toroviruses are members of the *Coronaviridae* family of single-stranded, positive-sense-strand RNA enveloped viruses. Their envelope is approximately 100 nanometers to 140 nanometers in diameter. The first incidence of detection of toroviruses in feces from patients with gastroenteritis occurred in 1984 (Beards et al., 1986). Subsequently, it was realized that toroviruses appear to be associated with persistent and acute diarrhea in children and may also be readily spread in a hospital (Koopmans et al., 1997; Jamieson et al., 1998).

Toroviruses are known causes of diarrhea among cattle, and identification in human specimens has been reported (Koopmans et al., 1993; Koopmans et al., 1997; Krishnan and Naik, 1997). Studies have also shown that torovirus may be associated with necrotizing enterocolitis (NEC) in newborn infants (Lodha et al., 2005).

2.2.9 The Coronaviruses

Coronaviruses are enveloped viruses with a positive, single stranded RNA genome of approximately 27 to 32 kilobasepairs. Prior to 2003, only 2 human coronaviruses were known – Human coronavirus (HCoV) 229E and HCoV-OC43 from the group 1 and group 2 coronaviruses respectively. Subsequently, a new strain of coronavirus, SARS-CoV, was discovered that is either a distinct member of the group 2

coronaviruses or the first member of a new group of coronavirus (Rota et al., 2003; Snijder et al., 2003; Gibbs et al., 2004). In addition, two other human coronaviruses have also been identified, HCoV-HKU1 and HCoV-NL63, that cause infections of the upper and lower respiratory tract (Kahn and McIntosh, 2005).

Coronaviruses are well-established causes of diarrhea in animals especially among swine, cats, mice, dogs and birds (Squires, 2003; Perlman and Dandekar, 2005; Weiss and Navas-Martin, 2005). The virus also causes respiratory disease in humans (Kahn and McIntosh, 2005). Coronaviruses are generally not considered to be a cause of acute gastroenteritis in adult humans as they have been detected in the stools of both diseased and healthy adults with equal frequency(Maass and Baumeister, 1983). They have, however, been shown to occur more often in the stools of infants and children with acute gastroenteritis than in healthy children (Maass and Baumeister, 1983; Kidd et al., 1989). Kern and colleagues have also shown that coronavirus excretion correlated significantly with the diagnosis of AIDS or with syndromes belonging to the AIDS-related complex though they could not establish whether the virus caused any disease in these individuals (Kern et al., 1985).

2.3. Virus Sampling and concentration methods for water

2.3.1 Concentration of Viruses from the Water Environment

The infectious dose of viruses is not precisely known but infections have been shown to occur with high probability even at very low doses (Bosch, 1998; Haas et al., 1999). Thus viruses are capable of causing disease even though they are present in the

environment in very low numbers. In order to obtain a representative sample, large volumes of water (10 to 1000 Liters) need to be concentrated down to smaller sample volumes for processing and virus detection methods (microliters to milliliters).. Guidelines published by the US EPA recommend that an equivalent volume of 10 Liters for sewage, 100 Liters for surface and ground-waters and 1000 Liters of finished water be analyzed in order to achieve a representative sample volume (Fout, 2001). Such large volumes of water would be unwieldy to store and to transport safely and at the right temperatures to the laboratory for processing. Several methods have been developed for the concentration of viruses from water - the virus adsorption/desorption (VIRADEL) method, cation coated filter method, and ultrafiltration. A number of methods have also been developed for the concentration of viruses following elution: organic flocculation, polyethylene glycol precipitation, and Speedvac concentration will be discussed.

2.3.2 Virus Adsorption Elution

The virus adsorption elution method (VIRADEL) was developed to allow the capture of viruses by passage of large volumes of water through electrostatic filters (Goyal and Gerba, 1983). Viruses are adsorbed to the filter surfaces, transported to the laboratory and then desorbed from the filters.

Some of the earliest filters used for the concentration of viruses consisted of cellulose fibres presented either as a membrane disk or a filter cartridge. The properties of cellulose acetate meant that when in use, they would adopt a negative electrostatic charge. Since most virus particles at neutral pH also tended to possess a net negative

charge, the use of these filters meant that the pH of the sample would have to be altered so as to impart a net positive charge to the virus particles and allow their adsorption to the filters. Commonly this meant lowering the pH of the sample to approximately 3.5 before passage through the negatively charged filter (Goyal and Gerba, 1983). The addition of multivalent cations has also been shown to facilitate virus floc formation and adsorption to the filters (Haramoto et al., 2004). The addition of varying concentrations of Manganese, Magnesium and Aluminum have all been proposed to enhance adsorption of viruses to the filters (Lukasik et al., 2000).

In order to avoid having to alter the pH of the water, which could potentially inactivate viruses (Darnell et al., 2004), positively charged filters were developed by Sobsey and his colleagues (1973). This also facilitated the collection of large volumes of sample water. At present, the list of commercially available positively charged filters include the 1MDS filter, Zeta Plus 50-s and 60s (Mehnert and Stewien, 1993; Kittigul et al., 2001).

Elution of the viruses is accomplished via one of several ways. 1%-2.9% Tryptose Phosphate Broth (pH 9.0) with or without glycine and arginine, 1.5% - 3% Beef Extract (pH 9.0 – 9.5) with or without glycine, 0.05M glycine (pH 10.5 – 11.5) and Urea-Arginine Phosphate Buffer (1.5M urea, 0.02M arginine, 0.008M phosphate; pH 9.0) have all been used as eluates with different filters to varying degrees of elution efficiency (Toranzos and Gerba, 1989; Jothikumar and Cliver, 1997; Kittigul et al., 2001; Dahling, 2002).

2.3.3 Cation coated filter method

The cation-coated filter method involves the precoating of conventional cellulose membranes with cations, commonly aluminium or magnesium followed by filtration, rinsing with 0.5M H_2SO_4 and elution with 1.0mM NaOH. Haramoto and colleagues have reported recovery efficiencies of greater than 80% using poliovirus-seeded milliQ water using this method (Preston et al., 1988; Haramoto et al., 2004; Haramoto et al., 2005). Cellulose membrane filters are inexpensive compared to the electropositive filters, however their use as reported thus far has only been for volumes of 10 liters or less.

2.3.4 Microfiltration and Ultrafiltration

Microfiltration and ultrafiltration involves the use of specially designed filters that are capable of denying passage to particles at the sub-micron size. Such filters have been used either directly, or indirectly to concentrate viruses from water and other environmental samples (Winona et al., 2001; Rutjes et al., 2005). In addition ultrafilters can also be used to recover all kinds of microbes, not just viruses which facilitates their use in assaying unknown microbial contamination events (Morales-Morales et al., 2003; Hill et al., 2005). The use of ultrafiltration to recover and concentrate viruses has been demonstrated at both the small ($\leq$ 2 liters) and at the large scale (100 liters) (Winona et al., 2001; Olszewski et al., 2005).

2.3.5 Organic Flocculation

Organic flocculation is the current EPA-recommended method for the concentration of viral particles following elution off filters. The addition of organic material like powdered beef extract has been shown to result in the formation of virus clumps or flocs around the larger organic molecule. This allows the virus to be pelleted by centrifugation at relatively low speeds (approximately 2500 x g). Organic flocculation has been used in a number of surveys to concentrate culturable viruses and bacteriophages from surface and groundwater, sludge biosolids and wastewater and has also shown to be compatible with subsequent downstream polymerase chain reaction detection methods (Pinto et al., 1993; Ma et al., 1994; Ma et al., 1995; Chapron et al., 2000; Chapron et al., 2000; Sedmak et al., 2003; Laverick et al., 2004; Sedmak et al., 2005).

2.3.6 Polyethylene Glycol Precipitation

Polyethylene glycol (PEG) precipitation has previously been reported to be more effective at concentrating some animal viruses compared to organic flocculation (Lewis and Metcalf, 1988; Boher, 1991). The addition of PEG is believed to sterically block the protein capsid of the virus from associating with the solvent molecules, resulting in the precipitation of the viruses, an alternative proposal is that the interaction of the protein charges on the virus with the PEG polymer causes crystallization to occur. It is believed that the actual mechanism of precipitation is the result of a combination of both of these processes (Lewis and Metcalf, 1988). The method has been used to detect rotaviruses, hepatitis A virus, Norwalk virus and adenoviruses in river, raw and treated drinking water

but is primarily used to detect viruses associated with foodstuff, especially shellfish (Hovi et al., 2001; Kingsley and Richards, 2001; Dubois et al., 2002; Goswami et al., 2002; Kingsley et al., 2002; van Zyl et al., 2004; van Heerden et al., 2005). A comparison of PEG precipitation and ultracentrifugation has shown PEG precipitation to be more effective at removing PCR inhibitors (Sunen et al., 2004).

2.3.7 SpeedVac Concentration

SpeedVac concentration of viruses has not been widely reported. The main proponents of this method for concentrating viruses have been a group from Mahidol University in Thailand. Kittigul and colleagues have reported a significantly higher rotavirus recovery when they compared speedVac concentration and PEG precipitation (Kittigul et al., 2001). The group has successfully detected poliovirus, hepatitis A virus and rotavirus from sewage and other environmental water samples (Kittigul et al., 2000).

2.4 Methods of Viral Detection

2.4.1 Cell Culture for Environmental samples

Cell culture is the most widely used method for the detection of enteric viruses concentrated from water. Cell culture methods exist for picornaviruses, rotaviruses, enteric adenoviruses, and astroviruses and are currently being developed for other viruses (Chapron et al., 2000; Pusch et al., 2005; Shieh et al., 2008). Unfortunately several of the newly emerging enteric viruses have not been amenable to culture, limiting the use of this technique. One of the viruses that had previously resisted attempts to culture are the

noroviruses. Straub et al. have successfully cultured noroviruses in a three dimensional fluid shear wall vessel bioreactor and postulate that previous attempts at culturing noroviruses failed due to the use of monolayers (Straub et al., 2007). Three dimensional cell culture systems are believed to be superior to two dimensional cell culture systems because they mimic the morphology and biochemical cell features that are present in the original cell tissue (Andrei, 2006).

2.4.2 Polymerase chain reaction (PCR)

The use of the polymerase chain reaction for the detection of pathogens was primarily a research tool that has rapidly gained acceptance in the clinical diagnostic setting. PCR has been shown to be an extremely sensitive, specific and rapid technique for the diagnosis of viral pathogens. The sensitivity of PCR has been demonstrated to be comparable or superior to cell culture (Bae et al., 2003; Raboni et al., 2003) though great care must be taken to avoid false positive reactions. The chief drawback of the PCR method is that it is incapable of distinguishing active and inactive targets. Prior to PCR, the viral nucleic acid must be extracted, several commercial extraction kits are available, most of them utilizing the Boom method developed by Boom and colleagues (Boom et al., 1999). One modification of the standard PCR method, Integrated Cell Culture PCR (ICC PCR), makes use of a cell culture step to enhance sensitivity and demonstrate infectivity. Another variant of PCR, Real Time PCR (RT PCR) can be used to quantify the original template concentration of the sample. Primers for the specific detection of many of the enteric viruses have been published (Beuret, 2004; He and Jiang, 2005; Jothikumar et al., 2005; Pang et al., 2005; van Heerden et al., 2005; Costafreda et al.,

2006; Elia et al., 2006; Oka et al., 2006; Yong et al., 2006; Young et al., 2006; Bird et al., 2007; Butot et al., 2007; Casas et al., 2007; Escutenaire et al., 2007; Haramoto et al., 2007; Xagoraraki et al., 2007; Nordgren et al., 2008).

2.4.3 Hybridization probes and microarrays

Dot-hybridization assays have been developed for adenoviruses and enteroviruses and have been used to detect quantities of target as low as 20 picograms of viral genome (Kidd et al., 1985; Takiff et al., 1985; Singh-Naz et al., 1988; Rosen et al., 1990; Fong et al., 2005). It can be useful for screening multiple samples against multiple targets but it is neither as sensitive nor as specific as PCR or cell culture.

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 either a hybridization of DNA, an immunological attachment or a chemical reaction can occur. Microarray technology is enabled by the ability to deliver sub-microliter volumes of material unto 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.

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 which serve to detect specific functional genes (e.g toxin producing genes; pathogenicity islands and catabolic enzymes) sequences irrespective of which species contains the sequence and phylogenetic arrays which target specific pathogens or groups of pathogen based on phylogenetic relationships.

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 2.2 summarizes some of the viral microarrays and their applications. Among the viral microarrays listed in table 2.2 seven array formats were developed for respiratory viruses, three were for various types of pox and pox-related illnesses (i.e VZV etc), three were for dengue and other vector-borne transmitted viruses and three were for enterovirus and other EV diseases (e.g Hand, foot and mouth disease, central nervous system diseases). In all cases, arrays were targeting viruses extracted from clinical samples. Of special interest were four arrays described as their developers as being universal or pan-viral arrays though only one system was actually shown to be able to detect a new unknown virus (Wang et al., 2003).

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 inch by 5 inch quartz wafer and are manufactured using a photolithographic masking technique (Pease et al., 1994). Agilent microarrays have a 44000 feature set and are synthesized using an inkjet printing method (Hughes et al., 2001). Febit's Geniom microarrays contain only 6 000 features but hybridization can be carried out with eight chips in parallel allowing multiple sample processing (Guimil et

al., 2003). Nimblegen microarrays contain 390 000 probes per array and are manufactured using a micromirror focusing technique, a technology borrowed from digital laser projectors in which tiny microscopic mirrors are used to target and focus beams of light on defined spots on the array, allowing a light dependent chemical reaction to occur on those selected spots. 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 routinely applied to the analysis of environmental samples.

Table 2.2 Viral Microarrays and Their Applications

Virus types	Numbers of gene Sequences	Application	Major Finding	Ref
Influenza A virus	12000 features / spots	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 oligoprobes 18-21 mer long	Distinguish 3 major circulating genotypes of VZV	Evaluated against 6 reference strains (OKA, YS, KEL.SD, MSP, BC) and 130 clinical specimens	(Sergeev et al., 2006)
Animal pestiviruses DNA suspension microarray	8 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	Approx. 22,000 oligo probes	Detected human parainfluenzavirus 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)	110 oligo probes	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 varicella-zoster virus (VZV), Herpes Simplex 1 virus (HSV-1), and Herpes Simplex 2 virus (HSV-2) that cause infections with clinical manifestations similar to OPV infections.	(Ryabinin et al., 2006)
Respiratory pathogen microarray ver 1	No data	20 common respiratory and 6 category A biothreat pathogens known to cause febrile respiratory illness	The results demonstrate a novel, timely, and unbiased method for the molecular epidemiologic surveillance of influenza viruses.	(Wang et al., 2006)

Table 2.2 continued

Pan viral DNA microarray, virochip	1592 probes, 25-mer	Virus discovery and identification	Identification of a Novel Gammaretrovirus in Prostate Tumors, and strongly implicated RNase L activity in the prevention or clearance of infection in vivo.	(Urisman et al., 2006)
four major serotypes of dengue virus	216 probes 22-mer	Detection and identification	host-blind probe design	(Putonti et al., 2006)
Flu-chip 55 diagnostic microarray	55 capture and label probes	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)

Table 2.2 continued

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)
Affymetrix resequencing Respiratory Pathogen Microarray (RPM v.1)	No data	species- and strain-level identification of respiratory viruses	Broad-spectrum respiratory pathogen surveillance	(Lin et al., 2006)
Varicella zoster virus expression microarray	71 probes 75-mer	Displays gene expression profile of VZV	Was able to show differences in levels of transcriptions among the various VZV ORFs	(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	23 different FMDV strains representing all seven serotypes were detected and typed by the FMD DNA chip.	(Baxi et al., 2006)
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)

Table 2.2 continued

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, herpes simplex virus type 1 and 2, varicella-zoster virus, human herpesvirus 7, human herpesvirus 6A and 6B, Epstein-Barr virus, polyomavirus JC and BK, cytomegalovirus, mumps and measles viruses		(Boriskin et al., 2004)

CHAPTER 3 RESEARCH QUESTIONS, HYPOTHESES, GOALS AND OBJECTIVES

3.1 Research Questions

Some of the research questions raised during examination of the current state of environmental virology that form the basis of this thesis are as follows:

- Do viruses represent a potential risk to swimmers in fresh waters such as at Great Lakes beaches?

- How can one determine the array of viruses present in polluted waters?

- Can new microarray technology be developed to characterize community viral infections by monitoring sewage?

- Can pathogen detection chip technologies like microarrays be used to determine which viruses represent the most likely source of risk based on their prevalence in the environment?

3.2 Research Hypotheses and Methodology

The premise of this study is that microarray technology can be adapted and used to perform high throughput characterizations of various water matrices for the common waterborne viral pathogens. In the absence of a defined list of target viral pathogen promulgated by the regulatory authorities or a suitable indicator for viral pathogens, microarrays are the next best option to screen complex environmental matrices for the hundreds of potential viruses that may be present. This dissertation will compare the current methods used to test for viruses in the environment, namely cell culture,

polymerase chain reaction (PCR) and integrated cell culture-PCR against the results obtained using a microarray to detect viruses through labeling and hybridization post cell culture. We will first characterize the types of viruses present at two typical recreational beaches in the Great Lakes impacted by human sewage using conventional cell culture and PCR-based methods. We will also compare the range of viruses present in raw sewage collected from a typical wastewater treatment plant using both polymerase chain reaction and the virus microarray and determine whether the virus microarray is able to detect a wider range of viruses in sewage compared PCR analysis of the same sewage.

3.3 Research Objectives

The objectives of this study were fourfold:

a. To use conventional cell culture and PCR based detection method to demonstrate the types of viruses that may be detected at recreational beaches that have been impacted by fecal pollution events.

b. To use freely available sequence information from online gene databases like GENBANK, to design virus specific probes targeting the known waterborne viral pathogens

c. To develop a standardized method for the concentration, extraction, labeling and hybridization of viral nucleic acid, from both DNA and RNA viruses, unto the viral microarray.

d. To characterize a human wastewater source using high throughput microarray analysis to screen for the presence of the target viruses of interest and

demonstrating that the kinds of viruses detected using a microarray are much greater than those detectable by conventional methods.

CHAPTER 4 EVALUATION OF PUBLIC HEALTH RISKS AT RECREATIONAL BEACHES IN LAKE MICHIGAN VIA DETECTION OF ENTERIC VIRUSES AND A HUMAN-SPECIFIC BACTERIOLOGICAL MARKER

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4.1 Abstract

In the United States, a total of 3003 days of beach closures and/or advisories were reported in the 2006 swimming season. Out of the 1445 beaches monitored, 336 were associated with high risk. In this study, water samples were collected along two Lake Michigan Beaches over the 2004 swimming season and examined for the presence of enteric viruses by cell culture and ICC-PCR. In addition, samples were also examined for the presence of a human sewage marker based on the Enterococcal Surface Protein (ESP). Results demonstrate that both beaches were impacted by human fecal pollution. Viruses were detected in 16/30 Silver Beach samples and 9/28 Washington Park samples, while the human specific ESP marker from cultivatable enterococci was detected in 21 out of 529 samples analyzed. The occurrence of bacterial-derived and viral indicators of human fecal pollution could not be correlated with each other. Predictive models of virus pollution were developed for both beaches utilizing physical parameters like wind speed, wind direction and water temperature. The best models for both beaches were determined with statistical support. Conclusion: Lake Michigan recreational beaches are being

adversely impacted by human fecal pollution. Monitoring for the traditional indicators of water quality does not address viral risks. Predictive models can be developed and potentially used as real time water quality forecasting tools. The risk of acquiring a viral infection at either of the beaches under study was determined to range from 0.2 to 2.4/1000 swimmers.

Key words: water quality; monitoring; predictive models; waterborne pathogens; fecal pollutants; indicators

4.2 Introduction

The Great Lakes are one of the world's largest bodies of freshwater. The volume of the Great Lakes is estimated at 6 quadrillion gallons and constitutes 95% of the United State's freshwater supply (GLIN, 2006). More importantly, the Great Lakes basin is home to more than 30 million people who use the water for drinking, recreation and industry. There are more than 1000 Great Lakes beaches along 5500 miles of shoreline located within the 8 US states and 2 provinces of Canada (Dorfman, 2006). The most recent National data in 2006, reported that the Great Lakes beaches exceeded standards 14% of the time and had the greatest exceedances compared to all other shorelines in the US (NRDC, 2007). There were 3003 days of beach closures and/or advisories for the 2006 swimming season and of the 1445 beaches monitored, 336 were identified as high risk associated with bacterial contamination and rain/runoff/stormwater however the greatest risks were associated with unknown sources.

It has been estimated that the twenty major cities located within the Great Lakes basin discharge in excess of 92 billion liters of raw sewage into the Great Lakes annually (MacDonald, 2006). The extent of impact to human health from the discharge of sewage into the Great Lakes has not been determined. A study in Southern California, a region that also experiences human health impacts from sewage discharged into recreational water bodies has estimated that there is between 627,800 and 1,479,200 excess gastrointestinal illnesses occurring at beaches in Los Angeles and Orange Counties each year and that this causes an estimated US$21 to $51 million worth of economic loss (Given et al., 2006). In comparison, Rabinovic et al. (Rabinovici et al., 2004) performed a study of the economic impact of beach closures on a Lake Michigan freshwater beach and found that a typical closure causes a net economic loss among would-be swimmers totaling $1274 – $37030 per day and between $111 088 and $518 415 over a 4 year period between 1998 to 2001 while avoiding 42% of the predicted illness.

The current US EPA recreational water guidelines are based upon the enumeration of enterococci and *Escherichia coli* (EPA, 1986). The guideline for freshwater is a geometric mean of at least 5 samples per month not exceeding 126 colony forming units per 100 ml (CFU/100ml) of *E. coli* or 33 CFU/100ml of enterococci. In addition, no sample should exceed a one-sided confidence limit (CL) of 75% CL for designated bathing beaches, 82% CL for moderately used beaches, 90% CL for lightly used and 95% CL for infrequently used beaches. Three of the biggest drawbacks of the current indicator methods as used for beach monitoring are the need to incubate the samples overnight, the inability to attribute the source of pollution and the lack of

correlation with pathogens of concern particularly viruses. Previous work has shown that there is a poor correlation between levels of viruses and bacterial indicators (Jiang et al., 2001; Noble, 2001). This is due to their different levels of excretion from the human body and their different rates of inactivation upon exposure to the environment (Tree et al., 2003). Examination of recreational water for the presence of viruses is currently not mandated by the ambient water quality criteria due primarily to the length of time needed to obtain results and also the cost of analysis. Also conventional cell culture-based methods do not provide the identity of the virus. These issues have been partly solved by the development of numerous PCR primers to allow the identification of the infectious viruses present in a sample using cell-culture-PCR methods (Chapron et al., 2000).

The polymerase chain reaction (PCR) has also been proposed as a means to rapidly and specifically detect microorganisms used in source tracking. A set of PCR primers has been reported by Scott et al. (Scott et al., 2005) to detect the presence of human enterococci using a specific marker for the enterococcus surface protein (*esp*) gene. Other library independent source tracking methods have been reported for *Bacteroides spp.* (Bernhard and Field, 2000) and *E. coli* (Ram et al., 2004). While the identification of human wastewater / sewage signatures aids in understanding the potential risk to swimmers, a report by the National Research Council has suggested that pathogen monitoring is needed to better define the probability of infection. In addition PCR is limited in regard to risk as it can not distinguish between viable and non viable microbial particles.

Many investigations into recreational outbreaks in freshwater systems fail to identify the etiological agents responsible, but frequently suggest that noroviruses, *Cryptosporidium* and *E. coli* might be causative agents. Surveillance of recreational waterborne disease in the United States from 1997 to 2004 showed that of the 94 recreational outbreaks recorded for untreated water, 28.7% were of an unknown etiology responsible for acute gasteroenteritis infections while *E. coli* spp. (24.5%), Norovirus (18.1%), *Cryptosporidium* spp. (10.6%), *Giardia* spp. (5.3%), and *Shigella* spp. (9.6%) were the most commonly identified microorganisms (Barwick et al., 2000; Lee et al., 2002; Yoder et al., 2004; Dziuban et al., 2006). Besides outbreaks, unknown gastroenteritis and generic diarrhea are the most common reported health outcomes in recreational epidemiological studies (Wade et al., 2006; Colford et al., 2007), and many believe these are associated with a variety of enteric viruses.

Wastewater has been suspected as the primary source of viral contamination of a wide range of ambient waters throughout the world. Patti et al. (Patti et al., 2003) evaluated 196 samples from various sources of water in Italy, detecting Coxsackie B virus in 35 and enteric- non-entero- viruses in 51 out of 196 samples screened. In Korea, Japan and the Netherlands untreated or inadequately treated sewage was the source of noroviruses and enteroviruses. Katayama et al. (Katayama et al., 2004) demonstrated the presence of enteric viruses (norovirus G1, G2, enteroviruses) in coastal seawater downstream of a Japanese wastewater treatment plant and up to 4 days following a combined sewer overflow event. A study in Korea by Lee et al. (Lee et al., 2004) found that thirty (75.0%) of the 40 samples collected from 4 rivers in a Korean province were

positive for enteric viruses based on cell culture. Loder and de Roda Husman (Lodder and de Roda Husman, 2005) were able to quantify a 2 to 100 fold dilution of norovirus and rotavirus in river water in the Netherlands using direct PCR impacted by wastewater. Both in Florida and California, adenoviruses were detected more often than enteroviruses in waters influenced by non-point sources such as septic tanks and stormwaters with acceptable levels of microbial water quality indicators (Wetz et al., 2004). An extensive survey of southern California urban waters by Jiang and Chu (Jiang and Chu, 2004) detected three types of human viruses (adenoviruses, enteroviruses and hepatitis A viruses) using nested- and RT-PCR from 11 rivers and creeks. Approximately 50% of the sites were positive for human adenoviruses and there was no clear relationship between detection of human pathogenic viruses and the concentration of indicator bacteria and coliphage.

Several recent epidemiological studies have examined new indicator systems for recreational water quality and their relationship to the health of swimmers. One of the larger studies took place in Mission Bay, California (Colford et al., 2007). Diarrhea and skin rash were related only to coliphage levels and traditional bacterial indicators were not adequate to address risk. The California study evaluated non-point source and storm water as the source of the pollution. In another study conducted in the Great Lakes, Lake Michigan and Lake Erie by Wade et al. determined that enterococci bacterial levels could be significantly related to gastrointestinal illnesses (Wade et al., 2003). In this case sewage was one of the likely sources. Time in the water was also associated with increasing illness rates as well.

The study described here addresses the public health risk associated with the degree of human fecal viral pollution at two Lake Michigan recreational beaches. Water samples were examined for the presence of viable enteric viruses through cell culture and integrated cell culture polymerase chain reaction (ICC PCR) and the application of a new human-specific enterococci bacterial marker was evaluated for its usefulness in addressing the presence of viral pathogens (Scott et al., 2005). This study was undertaken during the same time period that the epidemiological investigations were being implemented at Lake Michigan by the EPA for the National Epidemiological and Environmental Assessment of Recreational Water Study (NEEAR). (http://www.epa.gov/NEEAR/) , thus the results of this work will assist in meeting the goals of the BEACH Act in providing new assessment of pathogens, source tracking markers and risk assessment approaches for comparison to the traditional epidemiological investigations.

4.3 Materials and Methods

4.3.1 Area of study

Samples were collected from two public beaches located on Lake Michigan. Washington Park beach is located at 115 Lakeshore Drive, Michigan City, Indiana. The public beach measures approximately 3,500 ft. Sampling was carried out at a stretch of approximately 1,200 ft which experienced the most foot traffic (Figure 4.1). Three sampling points were chosen, approximately 400 feet apart. Silver Beach is located at St Joseph, Michigan. The public beach measures approximately 2000 feet. Sampling was

carried out at a stretch of approximately 900 ft which had the most foot traffic (Figure 4.2). Physical data for wind speed, wind direction from the Station MCYI3 buoy located at Michigan City, Indiana (41°43'45" N 86°55'41" W) was downloaded from "Michigan City Meteorological Data" located at http://www.glerl.noaa.gov/metdata/mcy/archive/mcy2003.07t.txt.gz (accessed on April 10, 2007) which is owned and maintained by the Great Lakes Environmental Research Laboratory. These data were used for both Washington Park and Silver Beach Park as it was the closest buoy. Temperature, pH and the number of people using the beach were recorded at the time of sample collection using a digital thermometer and pH meter. Turbidity data was recorded upon return to the laboratory by analyzing an aliquot of the sample on an Orion AQ4500 Portable Turbidity meter (Thermo Scientific Inc).

4.3.2 Sample Collection

Samples were collected from two public beaches along the shores of Lake Michigan (Figure 4.1). The sampling was performed in parallel with the EPA's National Epidemiological and Environmental Assessment of Recreational Water Study (NEEAR). In this study, samples for viruses and enterococci were collected every 2 weeks over ten weekends between July 3 and September 6, 2004. For virus analysis, a total of 58 samples were collected, each representing a composite of 3 spatial locations. At each spatial location, between 80 and 120 liters of lake water was first pumped into a sterile 20 gallon container, the sample's pH was lowered using 5N hydrochloric acid to produce an approximately neutral pH of between 7.0 to 7.5, the sample was then filtered through a 1MDS filer (Cuno, Inc) and the process repeated to give a sample volume total of

between 250 and 350 liters. All viral samples were held on ice, transported to the Water Quality and Health Laboratories at Michigan State University, and eluted within 72 hours. A total of 524 samples were analyzed for enterococci. All of the enterococci samples were processed through EPA contractors. Plates with membrane filters and colonies were shipped to MSU and kept at 4°C until processed.

Virus elution and concentration was carried out by the organic flocculation method as described by the US EPA information collection rule (EPA, 1995). Viruses were desorbed from the filters by two rounds of reverse passage of 1L of 1.5% beef extract solution (1.5% w/v Beef Extract, 0.05M glycine, pH 9.0 – 9.5). Viruses were flocculated by addition of ferric (III) chloride to a final concentration of 2.5mM and by lowering the pH of the solution to 3.5 (Payment et al., 1984). Viruses were pelleted by centrifugation at 2,500×g for 15 min and dissolved in 30ml of 0.15 molar sodium phosphate (final pH 9.0). Viruses were purified by centrifugation at 10,000×g for 10 min, brought to a neutral pH, supplemented with 100 units of Penicillin, 100 microgram of Streptomycin and 0.25 microgram of Fungizone and stored in aliquots at –80 °C until analysis.

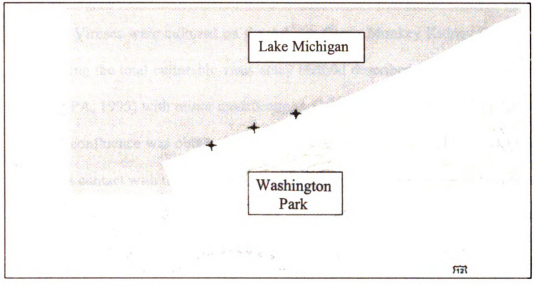


Figure 4.1. Overhead satellite image of the Washington Park sampling location. Stars indicate the sampling points.

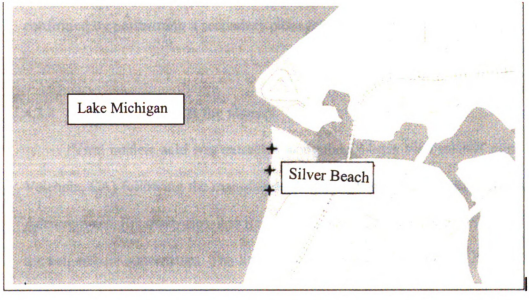


Figure 4.2. Overhead satellite image of the Silver Beach sampling location. Stars indicate the sampling points.

4.3.3 Cell culture

Viruses were cultured on the African Green Monkey Kidney (BGM) animal cell line using the total culturable virus assay method described in the information collection rule (EPA, 1995) with minor modifications. Cells were grown in flasks until at least 70% - 90% confluence was obtained. Virus concentrates were added to the flasks and allowed 2 hours contact with the cells with occasional rocking to ensure complete contact with the cells and to avoid drying of the cells. Concentrates were decanted and discarded and the cells were washed with Dulbecco's Phosphate Buffered Saline. Cells were maintained with Minimum Essential Media supplemented with L-glutamine, Earle's Salts and 2% Fetal Bovine Serum. Flasks were monitored for up to 14 days for development of Cytopathic Effects (CPE) indicative of a viral infection. Presence or absence of CPE was confirmed by performing a secondary passage for each flask.

4.3.4 PCR and RT PCR for viruses

Viral nucleic acid was extracted using the QIAgen viral mRNA mini kit (Qiagen; Valencia, CA) following the manufacturer's instructions. Primers developed to screen for Adenoviruses, Enteroviruses, and Rotaviruses were used to perform PCR and RT PCR on the cell culture supernatant. The list of primers is given in Table 4.1. For PCR reactions, thermocycler settings were as follows: 95 °C, 15 minute initial denaturation to activate the Hotstart Taq polymerase (Qiagen; Valencia, CA), 95°C, 0.5 minute denaturation, 57°C, 0.5 minute annealing and 72°C, 0.5 minute elongation for 35 cycles followed by a

final elongation step of 72°C for 5 minutes. The PCR mix consisted of 1 unit of Hotstart Taq polymerase, 1.5 millimolar $MgCl_2$, 1× PCR buffer, 1× Q solution, 0.5 micromolar of each primer, 0.5 millimolar of each dNTP. For the RT-PCR reactions, thermocycler settings were as follows: 50°C, 30 minute first strand synthesis, 95 °C, 15 minute initial denaturation to activate the Hotstart Taq polymerase (Qiagen; Valencia, CA), 95°C, 0.5 minute denaturation, 57 °C, 0.5 minute annealing and 72°C, 0.5 minute elongation for 35 cycles followed by a final elongation step of 72°C for 5 minutes. The reverse transcription PCR mixture consisted of 2 microliters of OneStep RT-PCR enzyme mix, 1.5 millimolar $MgCl_2$, 1× PCR buffer, 1× Q solution, 0.5 micromolar of each primer, 0.5 millimolar of each dNTP. PCR products were separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (BioWhittaker) and viewed under UV light.

Table 4.1. RT/PCR primers used in this study for the detection of viruses

Virus	RT/PCR or nested step	Primer	Sequence	Length (bp)	Reference
Enterovirus	RT/PCR	RT	ATTGTCACCATAAGCAGCCA	534	(Chapron et al., 2000)
		PCR	CGGTACCTTTGTACGCCTGT		
	nested PCT	E1	TCCGGCCCCTGAATGCGGCTA	138	
		E2	GAAACACGGACACCCAAAGTA		
Adenovirus	RT/PCR	hexAA1885	GCCGCAGTGGTCTTACATGCACATC	301	(Chapron et al., 2000)
		hexAA1913	CAGCACGCCGCGGATGTCAAAGT		
	nested PCT	hexAA1893	GCCACCGAGACGTACTTCAGCCTG	143	
		hexAA1905	TTGTACGAGTACGCGGTATCCTCGCGGTC		
Rotavirus	PCR	Fwd	TTGCCACCAATTCAGAATAC	211	(Borchardt et al., 2004)
		Rev	ATTTCGGACCATTTATAACC		

4.3.5 Extraction of Enterococci

The membrane filters and plates (mEI agar) that were used in determining the levels of Enterococci during the EPA's National Epidemiological and Environmental Assessment of Recreational Water Study on Lake Michigan were shipped to Michigan State University. The filters were initially extracted off the cellulose filter by lifting the membrane, suspending the membrane in tryptic soy broth (Difco), vortexing vigorously, and incubating the suspension for 2 hours at 41°C to wash the bacteria from the filters and partially enrich the culture. Following the incubation, the suspension was used as the environmental sample from which total DNA was extracted using a QIAamp DNA extraction kit according to manufacturer's instructions (Qiagen; Valencia, CA).

4.3.6 PCR Conditions for *esp* Marker

Primers specific for the *esp* gene in *Enterococcus faecium* have been previously developed and examined for specificity to human fecal pollution (Scott et al., 2005). The forward primer, which is specific for the *E. faecium esp* gene is: (5'-TAT GAA AGC AAC AGC ACA AGT T-3'). A conserved reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3') was used for all reactions.

PCR reactions were performed in a 50 microliter reaction mixture containing 1X PCR buffer, 1.5 millimolar $MgCl_2$, 200 micromolar of each of the four deoxyribonucleotides, 0.3 micromolar of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen; Valencia, CA), and 5 microliters of template DNA. Amplification

was performed with an initial step at 95°C for 15 minutes (to activate Taq polymerase), followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (BioWhittaker) and viewed under UV light. The polymerase chain reaction product is 680 base pairs in length.

4.3.7 Statistical Analysis

Multivariate analysis was performed to determine if there was any correlation between the observed data and the following predictors - wind speed, wind direction, water temperature, number of swimmers and turbidity recorded. The multiple regression model used had the following form:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 + \cdots + \beta_n x_n + \varepsilon \quad (1)$$

where the response y (most probable number / 100L) is represented as a combination of a constant, linear and interactive terms of the predictors $x_1, x_2, \cdots x_n$ and an error term ε. Given a set of n observations $(x_1, y_1), (x_2, y_2) \cdots$, equation (1) can be written in a matrix form whose solution gives the coefficients:

$$\boldsymbol{\beta} = \left(\mathbf{X}^{\mathbf{T}}\mathbf{X}\right)^{-1}\mathbf{X}^{\mathbf{T}}\mathbf{y} \quad (2)$$

where $\boldsymbol{\beta}$ and $\mathbf{y}$ are the column vectors of coefficients and the responses respectively. The design matrix $\mathbf{X}$ was based on equation (1) and contained in its columns the model terms evaluated at the predictors. One of the objectives of our analysis was to identify models

with the least number of parameters (β's) and the maximum explanatory power. We evaluated a large number of models and to aid model selection based on the principle of parsimony, we used the Akaike Information Criterion (AIC) defined as:

$$AIC = -2\ln(L) + 2k \qquad (3)$$

where L denotes the maximum likelihood estimation and k is the number of parameters (Akaike, 1974). Since the AIC penalizes likelihood with respect to the number of parameters, the idea of model selection is to select the model with the lowest AIC value. All computations were performed using MATLAB (Mathworks Inc., Natick, MA) and SYSTAT (Systat Software Inc., San Jose, CA).

4.4 Results

4.4.1 Viral results

A total of 30 and 28 lake samples were collected from Silver Beach and Washington Park respectively. Cultivatible viruses were detected by cell culture as evidence by cell death in 16 of the 30 (53%) water samples collected from Silver beach in St Joseph, Michigan and 9 of the 28 (45%) water samples collected from Washington Park beach, Michigan City, Indiana. Data for percentage positive, geometric mean, minimum-maximum MPN/100L, percentage positive morning and percentage positive afternoon data are summarized in Table 4.2.

Most probable number estimation of viruses ranged from <0.6 MPN / 100L to 4.33 MPN / 100L at Silver Beach with an average of <1.21 MPN/100L and between <0.5

MPN/100L and 5.70 MPN/100L at Washington Park Beach with an average of <1.30 MPN/100L. The limits of detection were used for non-detects in calculating the mean. Cell culture PCR or RT-PCR showed the presence of adenoviruses (53.3%), enteroviruses (3.3%) and rotaviruses (10%) at Silver Beach. For Washington Park beach, 39.3% and 7.1% of the samples were adenoviruses and enteroviruses positive through RT-PCR analysis of the cell culture supernatant.

4.4.2 Sequencing Confirmation

The presence of adenoviruses in the sample was confirmed through nucleotide sequencing. In total 5 samples (3 Washington Park samples and 2 Silver Beach samples) positive for adenovirus were selected at random and sequenced using the primers hexAA1893 and hexAA1905 (Table 4.1). Sequencing was carried out at the Michigan State University Research Technology Support Facility. Two different sequences were obtained from the 5 samples with a single nucleotide mismatch between them and showing approximately 99% sequence homology with Adenovirus type 40 viruses.

Table 4.2. Viral Water Quality at Lake Michigan Beaches

	Washington Park	Silver Beach	
Number of samples	28	30	
CELL CULTURE			
percentage positive for any virus by cell culture	32	58	
Geometric mean * MPN/100L	0.85	1.0	
Min-Max MPN/100L	<0.5-5.7	<0.6-4.33	
percentage positive morning samples	36	60	
percentage positive afternoon samples	29	47	
PCR			
Percentage positive by PCR	43	60	
Adenovirus percentage positive	39	49	
Enterovirus percentage positive	7	3	
Rotavirus percentage positive	0	9	
ESP percentage positive	32	15	

* non-detects used at limit of detection for mean

4.4.3 Enterococcus esp gene PCR results

A total of 294 out of 414 and 235 out of 414 enterococcus membrane filters were analyzed for the presence of the ESP gene sequence from Silver Beach and Washington Park respectively. While virus samples were collected twice a day at three near shore sampling points, Enterococcus samples were collected thrice a day at 6 sampling points, 3 corresponding to the virus sampling points and 3 in further out away from shore. Eight Silver Beach (32%) and thirteen Washington Park samples tested positive for the *esp* gene in at least two separate PCR reactions. Analysis of the frequency of detection of the *esp* gene was not found to correlate significantly with the presence of either PCR-detectable or culturable viruses. A Chi-square comparison of ESP PCR results and viral PCR results generated a Chi value of 1.89 with 1 degree of freedom resulting in a failure to reject the null hypothesis that the ESP and viral PCR results are independent at the 95% confidence level.

4.4.4 Virus PCR and Cell Culture results correlation

Most probable number estimations of virus using cell culture results were compared with virus PCR results to determine if a positive cell culture result as evidence by cell death was statistically more likely to give rise to a positive virus PCR result. Chi square analysis of the combined virus data for both beaches generated a Chi square value of 4.66 with 1 degree of freedom. Therefore it was concluded that at the 95% confidence level that a positive cell culture result was statistically more likely to correlate with a positive virus PCR result. A similar analysis of Silver Beach samples at the 95%

confidence level likewise showed that a positive cell culture result was statistically more likely to correlate with a positive virus PCR result. Results were inconclusive for Washington Park data however as the expected value obtained was less than five in some cases, which violated the conditions for performing a reliable Chi square test. From the statistical tests, we can conclude that a positive PCR result correlates with a positive cell culture result and vice versa.

4.4.5 Physical Indicators

Average pH, water temperature, wind speed, wind direction, turbidity and number of swimmers recorded for both beaches are provided in table 4.3. At Silver Beach, which is located approximately 40 miles northwest of Washington Park beach, the average turbidity and number of swimmers was found to be greater than at Washington Park Beach. The pH, water temperature and windspeed were found to be similar at the two beaches. The prevailing wind direction for Washington Park beach was found to be directed mostly towards the shore while the prevailing wind direction for Silver Beach was generally directed along the shoreline.

Table 4.3. Average pH, water temperature, wind speed, wind direction, turbidity and number of swimmers recorded for Washington Park and Silver Beach samples. Range is given below in parentheses

Sample Location	Average pH	Average turbidity (NTU)	Water Temp / °C	Windspeed (m/s)	Wind direction (0° = true north)	Number of swimmers
Washington Park	8.4 (8.20, 8.72)	2.71 (0.5,13.9)	22.1 (20, 25)	2.40 (0.88, 3.82)	93.9 (195, 250)	17.75 (0, 75)
Silver Beach	8.46 (8.27, 8.67)	3.45 (0.3, 16)	21.6 (18.1, 24.5)	2.37 (0.60, 4.78)	49.6 (194, 165)	39.8 (0, 180)

4.4.6 Modeling Virus Contamination at the Beaches

Wind direction is an important variable for beaches situated near river outfalls or storm drains (Nevers and Whitman, 2005; Liu et al., 2006). However, the influence of wind on beach water quality can be complex and can vary depending on the orientation of the beach (θ_{Beach}) relative to the geographical north and the prevailing wind vector. For example, if the wind direction is from the north to the south (onshore) and is perpendicular to the orientation of the beach (East to West), then the river plume can be forced onshore and travel to the nearby beaches along the shoreline. The opposite is true for southern winds which may push the river plume out into the lake causing dilution. Wind directions are generally reported relative to the north (e.g., 0° = wind coming from the north, 90° = wind coming from the east etc.).

An accurate assessment of the influence of wind direction for different beaches requires that the effect of the wind direction be evaluated relative to the orientation of the

beach (Nevers and Whitman, 2005). Therefore, instead of using the wind direction (angle) as an independent variable in our models, we used a new variable (wind direction code) that took one of three possible values depending on the action of the wind in the near-shore region as shown in Figure 4.3 (i.e., onshore winds , offshore winds , and alongshore winds aiding or opposing the prevailing long-shore current component).

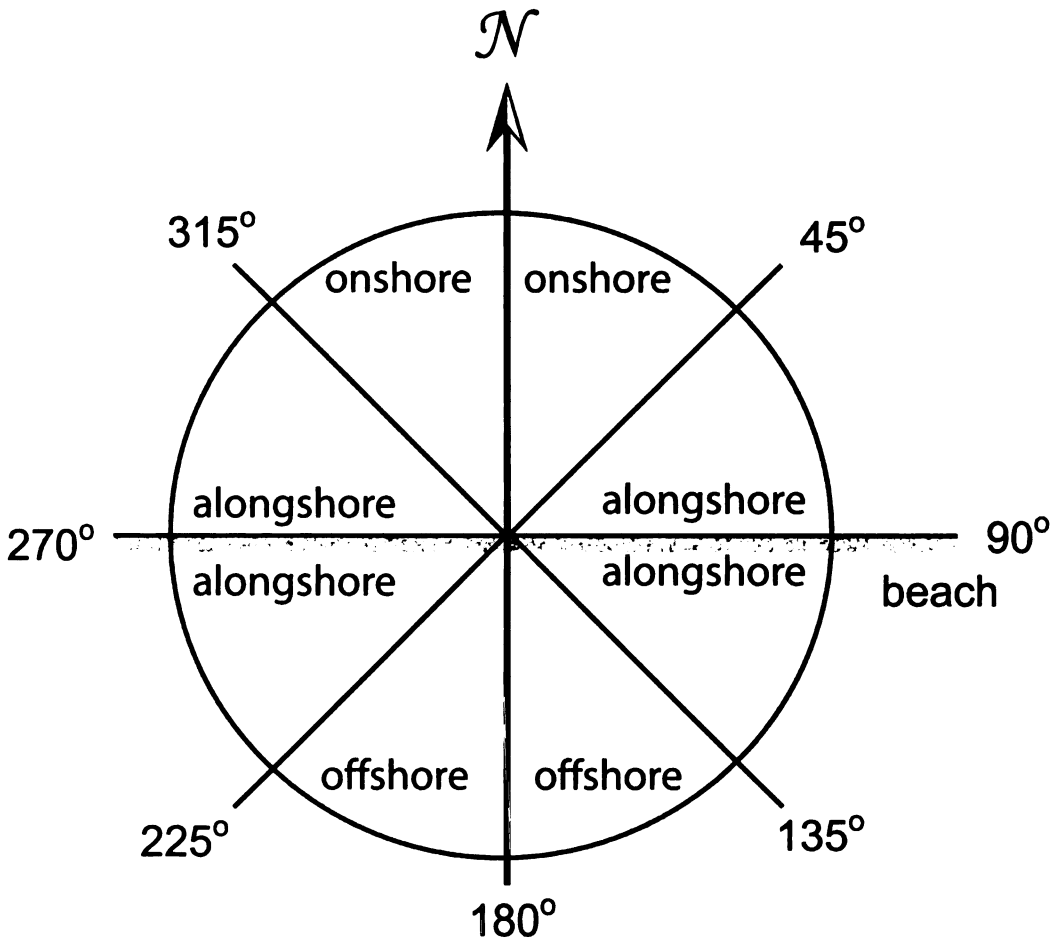


Figure 4.3. The effect of wind direction in the near-shore region depends on the orientation of the beach. Here the beach is oriented in the East-West direction (θ_B = 90 °C) and so a Northerly wind would blow in the onshore direction. In a beach oriented in a North-South direction, the same Northerly wind would be blowing in an alongshore direction. When the beach angle θ_B changes, then the angles corresponding to the onshore, offshore and alongshore winds will also change. For Silver Beach and Washington Park, θ_B = 205°, 72° respectively.

Multiple regression results (R^2 and adjusted R^2 values) indicated that the new variable (Beach orientation, θ_{Beach}) significantly improved the ability of the model to explain the observations at the two beaches. θ_{Beach} values for Silver Beach and Washington Park are 205^o and 72^o respectively (measured from the geographical north, positive clockwise). For Silver Beach, wind vectors between 250 - 340^o correspond to onshore winds, offshore winds occur between 70 and 160^o and alongshore winds for the other directions (341 - 69^o and 161-249^o). Values of the wind direction code variable used in multiple regressions were 1.0 for onshore winds, 0.0 for offshore winds and 0.5 for alongshore winds. An important consideration was to ensure that the variables included in the model are not redundant or collinear. Collinearity problems were identified (and resolved) by examining the eigenvalues of the $(\mathbf{X}^T\mathbf{X})$ matrix in equation (2). Models for both beaches were found to have condition indices significantly below 15 indicating that collinearity was not an issue (Belsley et al., 1980).

Water temperature was an important predictor; however missing values in the dataset make it difficult to compute the correlations with confidence. The Bartlett chi-square test which tests a global hypothesis about the correlations was significant ($p <$ 0.03) for Silver Beach which indicated that there may be some real correlations among the variables. For analysis with missing data, we used the EM (Expectation Maximization) algorithm to compute the correlations. For Silver Beach, water temperature significantly (and negatively) correlated with the virus data (Most Probable

Number, MPN) followed by turbidity, wind speed and wind direction. The number of swimmers correlated least with the viral data. A scatter plot matrix of the associations between the variables is shown in Figure 4.4 for both beaches with normalized density plotted along the diagonal of the matrix. The standard deviations of the variables determine the major axes of the confidence ellipses in Figure 4.4.

For Washington Park, turbidity showed the strongest association with MPN and wind direction and turbidity were related, however the Bartlett chi-square test failed due to the small sample size. Pearson correlations based on Bonferroni-adjusted probabilities (which provide protection for multiple tests) showed that wind direction and turbidity were related ($p < 0.001$) for Washington Park. Durbin-Watson statistic for all models showed that there was no evidence of autocorrelation. The best models for Silver Beach (R^2 =0.92, AIC = -5.25) and Washington Park (R^2 =0.99, AIC = -17.86) are given below.

Silver Beach:

$$\log_{10} y = 2.760 - 0.117*(\text{Water Temperature}) - 0.019*(\text{Turbidity})*(\text{WindDirCode}) \quad (4)$$

Washington Park:

$$\log_{10} y = 6.208 - 0.303*(\text{Water Temperature}) + 0.369*(\text{Turbidity})*(\text{WindDirCode}) \quad (5)$$

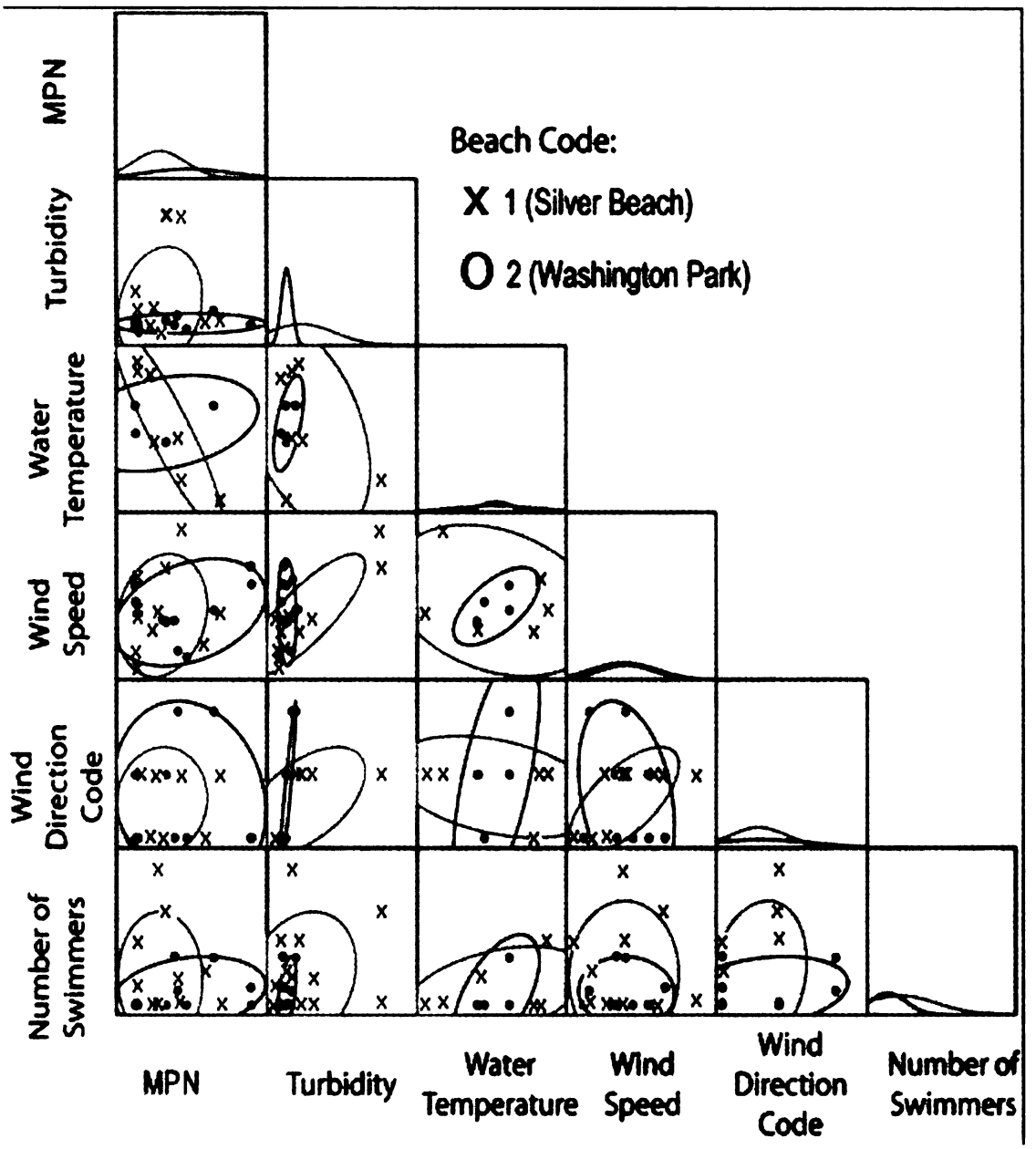


Figure 4.4. Scatter plot matrix showing the association between the variables for the two beaches. A narrow ellipse indicates stronger correlation. The orientation and major axes of the confidence ellipses depend on the standard deviation of the variables.

Computed t-values for all model parameters were significantly greater than 2.0 which was an indication that the independent variables were selected without a high degree of correlation among them (important for estimating the regression coefficients with confidence).

Models that best describe the observed variability at the two beaches (equations 4 and 5) were selected based on the principle of parsimony (AIC) and were found to contain an interaction term between turbidity and wind direction. Turbidity in the near-shore region is often an indicator of high suspended solids which may transport (depending on the wind direction) a variety of biological agents including viruses and bacteria. A comparison of the observed data and the model predictions (Figure 4.5) shows that a good agreement was obtained. The high R^2 values (0.89 and 0.98) indicate the goodness of the fit; however, these numbers can be expected to decrease as the sample size increases – typical R^2 values for beach models in the Great Lakes region range from 0.4 to 0.7 (Francy et al., 2003; Olyphant and Whitman, 2004). Although the present analysis was somewhat limited due to the small sample size of the data and the missing values, we were able to extract the important predictors and obtain a relation between them. The models were able to explain a majority of the observed variability at the two beaches.

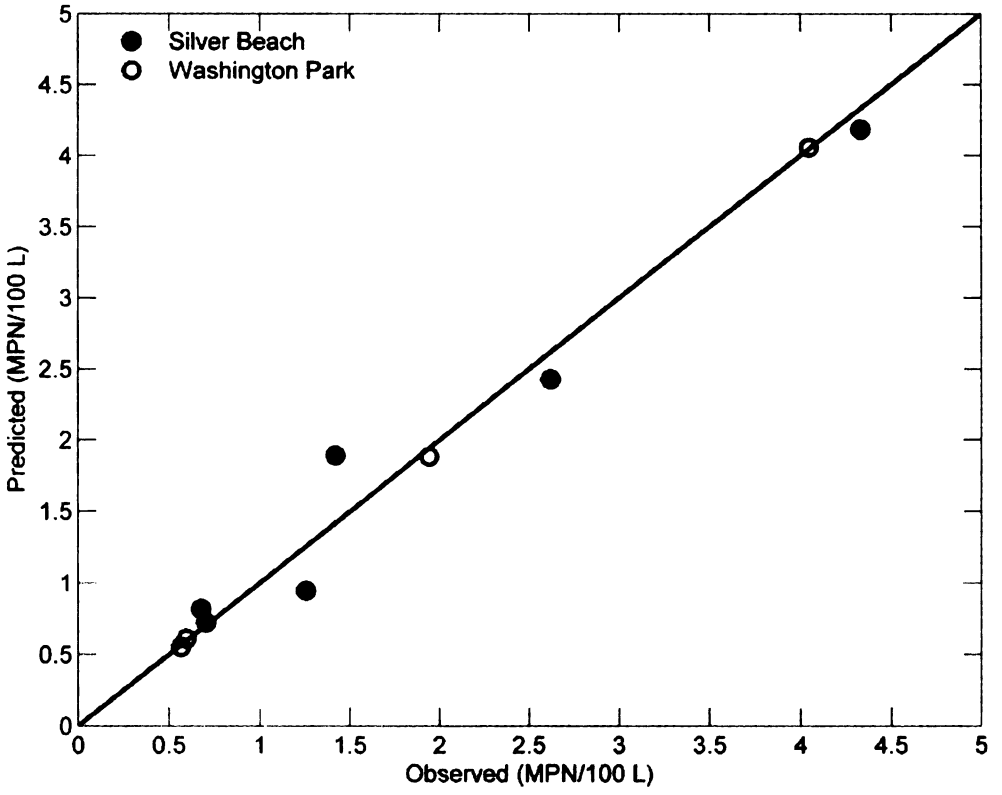


Figure 4.5. Observed and simulated viral data plotted on a 1:1 line for Silver Beach and Washington Park.

4.4.7 Risk Estimation

Using the virus MPN data and an exponential probability of infection model [$Pi = 1-e^{-rN}$] with a r value of 0.4172 (Crabtree et al., 1997) the daily probability of infection for an assumed dose of 100ml was determined. The daily risk of infection for Washington Park ranged from 0.24 per 1000 swimmers to 2.4 per 1000 swimmers. Similarly for Silver Beach, the probability of infection was determined to range from 0.26 per 1000 swimmers and 1.8 per 1000 swimmers when culturable viruses were detected.

4.5 Discussion

4.5.1 Overall Discussion of Results

Two beaches on Lake Michigan were assessed over a summer season for human fecal pollution and potential public health risks using two cultivation methods, one for viruses and one for a genetic marker (*esp*) detected within culturable Enterococci which indicates human sewage. The detection of cultivatable viruses and the detection of the *esp* gene marker indicated that both of these beaches have been impacted by human fecal pollution.

The lack of association between the *esp* gene marker and virus PCR results highlight the continued problems of using indicator systems to address pathogens. However, while *esp* and the virus data seem to give contradictory indications as to which beach was most heavily impacted by human fecal pollution, it is interesting to note that

probability of infection estimates based on the virus MPN values do seem to agree with the *esp* gene data in indicating that Washington Park poses a slightly higher risk for swimming.

The increased presence of the *esp* at Washington park (32% positive) was also reflected by the presence of enteroviruses (7% positive), compared to 15% and 3% positive, respectively at Silver Beach. However, adenoviruses were much more prevalent and showed an opposite trend. Previous research has reported an inactivation rate constant, k, for Enterococci of approximately 1.5 d^{-1} for concentrations of less than 50 colony forming units (CFU)/100 milliliters while an inactivation rate constant, k, of less than 0.5 d^{-1} was observed for concentrations above 50 CFU / 100 milliliters (Liu et al., 2006). No comparative data on sunlight inactivation for adenoviruses in water are available. However, in UV-inactivation laboratory studies, adenoviruses were demonstrated to be more resistant compared to coliphages, feline caliciviruses and enteroviruses (Thurston-Enriquez et al., 2003). Inactivation rate constants for adenovirus were reported to range between 0.018 to 0.040, feline and bovine caliciviruses were reported to range between 0.106 and 0.293, enteroviruses were reported to range between 0.119 to 0.181, coliphages were reported to range between 0.055 to 0.396 and Enterococci were reported to have an inactivation rate of 0.312 (Hijnen et al., 2006). Thus a valid hypothesis is that the Enterococci and enteroviruses are dying off much more quickly than the adenoviruses.

4.5.2 Modeling of Pathogens and Indicators

Previous attempts to model Lake Michigan beaches to relate fecal indicator levels with physical parameters have all enjoyed a measure of success. Nevers and Whitman showed that their regression model, utilizing parameters like wind direction, wave height, turbidity, lake chlorophyll, was able to predict *E. coli* levels and forecast closures more accurately than the current monitoring scheme (Nevers and Whitman, 2005). Olyphant and Whitman evaluated a number of different physical parameters and finally determined that wind direction, wind speed, rainfall, insolation, lake stage, water temperature and turbidity was able to accurately predict the geometric mean *E. coli* concentration in the swimming zone of a Lake Michigan beach (Olyphant and Whitman, 2004). Their model accounted for 71% of the observed variability in the log *E. coli* concentration. More importantly, their model was able to accurately predict openings versus closings 88% of the time. In our study, we showed that predictive modeling could be used to estimate the levels of actual pathogens. The Most Probable Number (MPN) values for viruses at Silver Beach was found to be dependent on water temperature followed by turbidity, wind speed and wind direction respectively. For Washington Park, turbidity showed the strongest association with MPN then wind speed and then wind direction.

4.5.3 Significance of Detection Methods

The current study was based on cultivation followed by PCR for both viruses and Enterococci *esp*. Direct PCR can potentially improve the speed by which analyses is undertaken but may affect accuracy as well as fail to address issues of infectivity. Quantitative methods can begin to address the level of contamination. Realtime PCR

analysis of the samples collected in this study for the presence of adenoviruses by Xagoraraki et al (Xagoraraki et al., 2007) showed that Silver Beach was also more contaminated with 60% (35 samples) of the adenovirus cultivation-PCR analyses in agreement with the real-time adenovirus PCR results. In order to choose the best method one must address a number of issues. Firstly, cell culture typically examines a much larger volume of analyte compared to PCR, in some cases leading to enhanced sensitivity, however PCR is able to detect targets at much lower concentrations compared to cell culture. Secondly, cell culture is able to address the issue of viability which PCR lacks and is an important factor in assessing human health impacts. Thirdly, PCR and related methods are able to better assess a wider range of targets compared to cell culture which are generally restricted in terms of targets. Lastly, in situations where the time-to-result is crucial, PCR and PCR-related methods are much faster than cultivation methods.

4.5.4 Adenovirus Presence

The detection of adenovirus gene sequences in this study indicates that there exists a risk of adenovirus infection while swimming and wading. Waterborne outbreaks caused by or associated with human adenoviruses have been documented, but mostly in recreational swimming pools (Turner et al., 1987; Papapetropoulou and Vantarakis, 1998; van Heerden et al., 2005). Even though adenoviruses are included in the US EPA's candidate contaminant list, few studies have looked into the occurrence of human adenovirus in freshwater recreational beaches. This study indicates that adenoviruses were the most frequently detected enteric viruses among the different virus types screened and that adenoviruses might make a good indicator of viral fecal pollution. A

more comprehensive survey of multiple freshwater locations needs to be carried out to determine if adenoviruses are similarly prevalent at other recreational locations.

4.5.5 Future Research Needs

There might also be other suitable viral indicators of human fecal pollution that await discovery. Unfortunately we currently lack adequate tools to perform high throughput screening of environmental samples in order to characterize the virus signatures that might be present. To date, several sets of primers have been published for the detection of human enteric viruses however multiple pathogen detection of viruses remains an elusive goal. Current research focusing on developing multiple pathogen screening tools like microarray technology might be able to eventually allow environmental samples to be screen for many multiple viruses in a cost effective and rapid manner.

4.6 Conclusions

- Both these recreational beaches along Lake Michigan were shown to have been exposed to human fecal pollution at the time of the study.
- The risk to swimmers at the beach was determined to be low but present.
- The presence of the viral and bacterial indicators used in this study did not correlate with each other, demonstrating a possible deficiency in the current bacteria-based monitoring system to address viral sources of contamination.

- Adenoviruses were determined to be the most prevalent enteric virus detected at these beaches as detected by both cell culture-based PCR and real-time PCR with a high degree of agreement between the two systems.
- Statistically supported predictive models could be constructed for the two beaches using the viral data and physical parameters. Similar models have been developed and implemented at other recreational beaches for the determination of risk to swimmers and bathers.

CHAPTER 5 DETECTION OF PATHOGENIC VIRUSES CIRCULATING IN COMMUNITY WASTEWATER USING OLIGONUCLEOTIDE MICROARRAYS

By Mark Wong, Syed A. Hashsham, Erdogan Gulari, Jean-Marie Rouillard, Joan B. Rose

Submitted for consideration to Environmental Science and Technology

5.1 Abstract

This study describes the novel use of a viral microarray to screen municipal wastewater for the presence of circulating human pathogenic viruses. A total of 780 unique probes targeting 27 different groups of both DNA and RNA viruses were designed and tested against laboratory strain viruses and environmental water samples. Approximately thirty probes were used to target each viral group. Laboratory strains of poliovirus and adenovirus type 40 and 41 were evaluated initially and indicated that the probes were highly specific for their targets and that cross hybridization of target nucleic acid was minimal even when closely related virus species were mixed and co-hybridized on the array. During 13 months of sampling, RNA viruses were more frequently detected in a community wastewater compared to DNA viruses. Overall, more viruses were detected during the winter season compared to the summer months. Conclusion: Microarrays are capable of screening for a broad number of pathogenic viruses which may be circulating in the population and excreted in the community wastewater. This is the first demonstration of an environmental microarray for detection of viruses in water and could be used to improve public health surveillance.

5.2 Introduction

Viruses remain one of the most significant groups of human pathogens associated with global disease in the 21st century. While smallpox has been eradicated and vaccines are available for several viruses, resurgence of poliovirus in the developing world and the rapid spread globally of newly evolving viruses such as SARS and new strains of influenza have demonstrated that characterization and detection remain important for defining public health strategies in the future (Tambyah, 2004; 2006; Wong et al., 2007). Enteric viruses, including many groups in the picornaviradae, reoviradae and adenoviradae family also remain a significant cause of global diarrhea, neurological, respiratory as well as other chronic conditions associated with hepatitis and myocarditis (Glass et al., 2001; Leclerc et al., 2002; Oberste and Pallansch, 2003). The enteric noroviruses are associated with major outbreaks on cruise ships, at restaurants and in nursing homes with a global distribution of new more pathogenic types. Recently, it has been suggested that high viral excretion loads of Norovirus (NoV) Genogroup II in fact is associated with its global predominance (Bull et al., 2006). A high infant mortality (600,000 deaths annually) is still associated with rotavirus infections in the developing regions of the world (Parashar et al., 2003).Adenoviruses have also been found to be of significant concern causing gastroenteritis, upper and lower respiratory infections, conjunctivitis. acute and chronic appendicitis, cystitis, exanthematous disease, and nervous system diseases (Xagoraraki et al., 2007). Currently, it is estimated that over 150 types of viruses may be excreted in human and animal waste (Tartera and Jofre, 1987). But a true catalog of the numbers and types of viruses remains elusive. In large part, this

is due to the lack of tools with which to screen environmental samples for the presence of human-pathogenic viruses in a high throughput, high efficiency manner.

At present, cell culture in combination with molecular methods or polymerase chain reaction (PCR) alone have been used for detection of pathogenic viruses in water (Metcalf et al., 1995). Nevertheless, it is believed that many viruses exist that are refractory to cell culture (Seymour and Appleton, 2001). Even if successfully cultured, cell culture alone still provides no indication as to the virus' identity. PCR has improved the sensitivity and specificity of viral analysis of clinical or environmental samples. However it has been difficult to design multiplex primers for more than 5-8 targets in a single reaction, invalidating its use as a primary screening tool without some knowledge as to the potential viral etiology. Secondly, PCR is incapable of targeting new and emerging infectious viruses for which no known primers exist.

In recent years, virus microarrays have been developed and used to detect and characterize pathogens from clinical samples allowing for broad screening and pathogen discovery. Microarrays have been designed to detect and genotype Hepatitis B virus, adenoviruses, Epstein Barr Virus, Herpes Simplex virus, influenza virus and human papillomavirus (Wang et al., 2002; Wilson et al., 2002; Sengupta et al., 2003; Wang et al., 2003; Boriskin et al., 2004; Korimbocus et al., 2005; Baxi et al., 2006; Chen et al., 2006; Chiu et al., 2006; Liu et al., 2006; Min et al., 2006; Putonti et al., 2006; Song et al., 2006; Lin et al., 2007; Wong et al., 2007). For this and other reasons, microarrays are

being developed and used as a clinical screening tool to detect and subtype a wide array of viruses (Wang et al., 2002).

One potential application of microarrays as pathogen screening tools is their use as biosensors for pathogens in the environment. To date however only a handful of examples of environmentally-applied pathogen detection microarrays have been reported (Call et al., 2003; Rhee et al., 2004; Sergeev et al., 2004; Wu et al., 2004; Maynard et al., 2005; Lee et al., 2006; Kostic et al., 2007; Quinones et al., 2007; Miller et al., 2008). One reason for the dearth of examples of environmentally applied microarrays is the presence of inhibitory substances in the environment that compromise sample labeling and hybridization efficiency. Another reason is the lack of sufficient sensitivity to detect the low concentration of the target pathogens in relation to non-targets in the environment (Straub and Chandler, 2003; Wagner et al., 2007).

The aim of this project was to design, evaluate and demonstrate the use of microarrays for the detection of pathogenic viruses in sewage. Through this characterization of circulating pathogens within a community one can begin to address broadly an approach for biomonitoring and the screening of community health.

5.3 Materials and Methods

5.3.1 Viruses and Wastewater Sampling

Poliovirus LS-C-1, Adenovirus type 40 and type 41 were obtained from the American Type Culture Collection (ATCC # VR-59, VR-931 and VR-930 respectively).

All viral infections were performed using the African Green Monkey Kidney cell line - BGM, which were cultured in DMEM supplemented with 10% Fetal Calf Serum and antibiotics. Viral infections were allowed to proceed until the onset of cytopathic effects (typically within 24 hours). To recover the viruses, infected cells were freeze-thawed three times to disrupt the cell integrity and release the viral particles into the cell culture media. Virus particles were recovered and concentrated from the media by centrifugation through an Amicon Ultra 100K centrifugation column (Millipore, Billerica, MA). Virus nucleic acid was extracted using the QIAamp viral RNA mini kit for both DNA and RNA viruses following the manufacturer's instructions. The QIAamp viral RNA mini kit has previously been demonstrated to be capable of isolating viral DNA as well as viral RNA(Kleines et al., 2003). Briefly, 140 microliters of virus concentrate was added to tubes containing 560 microliters of lysis buffer and incubated at room temperature for 10 min with intermittent mixing. 560 microliters of ethanol was added and the solution mixed before passage through a DNA binding column. Columns were washed with washing solution and eluted with 2 rounds of elution solution using 50 microliters of DNase and RNase free water each time.

Thirteen 6 liter samples of untreated sewage were collected from August 2006 and September 2007. Samples were brought back from the laboratory and 15% buffered beef extract (Difco Inc) was added to give a final concentration of 1.5% beef extract and 0.05 molar glycine. The solution was stirred for 30 minutes before 2.5 molar $FeCl_3$ was added to a final concentration of 2.5 millimolar. The pH of the solution was lowered to 3.5 and the solution was stirred a further 30 minutes. Viruses were pelleted by

centrifugation at 2,500 x g for 15 minutes. The viral pellet was resuspended in 90 milliliters of 0.15 molar sodium phosphate dibasic (pH 7.0) solution by agitation on an orbital shaker set to 160 revolutions per minute. Once the pellet was dissolved, the pH of the sodium phosphate was raised to between 9.0 and 9.5 and placed on the orbital shaker for a further 10 minutes. Solid particles were pelleted by centrifugation at 10000 x g (Beckman model J2-HC). The supernatant was collected and filtered through a 0.22 micrometer syringe filter and supplemented with 100 U/ milliliter of Penicillin, 100 microgram/ milliliter of Streptomycin and 250 nanogram/milliliter of Fungizone. The pH of the virus concentrate was neutralized to 7.0 and frozen at –80 °C until placed on cell culture.

Twelve milliliters of virus concentrate was used to infect BGM cells grown to approximately 80 – 90% confluence. Virus concentrate was allowed 120 minutes of contact with the BGM cells before being discarded. Cells were incubated at 37 °C until development of cytopathic effects were observed. Infected cells were harvested by mechanical lifting using a sterile cell scraper. All cells and free viruses in the media were concentrated by centrifugation through an Amicon Ultra 100K centrifugation column (Millipore, Billerica, MA). Virus nucleic acid was extracted using the Qiagen viral RNA mini kit in the same manner as described above for the ATCC strains of viruses.

5.3.2 Microarray Design and Construction

Viral sequence data were obtained from the curated database of fully sequenced viral genomes in GenBank. Seventeen RNA virus groups and 10 DNA virus groups were

targeted. A list of sequences used to design probes is given in Table 5.1. Genome sequences were parsed through the Oligoarray 2.1 (Rouillard et al., 2003). The probes were designed to conform to the following specifications: maximum melting temperature (Tm) = 75 °C (except for torovirus = 80 °C), minimum Tm = 70 °C (except for torovirus = 65 °C); maximum guanine + cytosine (GC) content = 60%, minimum GC = 40%, maximum temperature for secondary structure = 45 °C, maximum temperature for cross hybridization = 45 °C. Probes were designed from the positive strand of the genetic sequence. A total of 780 specific probes were designed (approximately 30 probes per viral family target). Generating multiple probes for each target family was thought to enhance the reliability of detection by providing multiple binding sites on the chip for target hybridization.

Microarray chips were synthesized by using the in situ synthesis technology developed by the University of Michigan as described previously (LeProust et al., 2000; Gao et al., 2001; Komolpis et al., 2002). The microarray chip format used for the virus chip has a 7 column 10 by 100 array with a potential for containing a maximum of 7000 spots. Spots were randomly populated with 5 copies of each of the 810 designed probes representing 48% of the chip capacity. Multiple copies of probes were used to provide technical replication of the signals. The complete microarray probe layout and annotated probe information has been deposited at the Gene Expression Omnibus (GEO Accession # GPL6501). The microarray substrates were made by etching a continuous serpentine channel ~ one millimeter wide, seventy microns deep and 10 mm long on a 10 mm x 14 mm silicon substrate. The glass cover was bonded to the top of the silicon substrate to

provide a closed channel for the in situ synthesis of the oligos. After derivatizing the silicon and glass surfaces with an aminosilane linker a 15 thymine spacer was synthesized on top of the linker to reduce the steric hindrance. The final 25-28 mer probes were in situ synthesized using the phosphoramidite chemistry modified to work with photogenerated acid. Recirculation of the sample in the closed continuous serpentine channel of the microarray ensured good exposure of all the probes to the DNA and RNA targets in the sample during hybridization and reduced the time to reach equilibrium hybridization. The flow of the sample solution also helps remove weakly held mismatched targets. The closed nature of the microarray also totally eliminates any bleaching related to atmospheric ozone.

Table 5.1. List of viral targets, type of genomes and Genbank accession numbers

Virus	Type of genome	Accession no.	Sequence length (bp)
Hepatitis A virus*	ssRNA positive no DNA stage	NC_001489	7478
Hepatitis E virus*	ssRNA positive no DNA stage	NC_001434	7176
Human adenovirus A*	dsDNA	NC_001460	34125
Human adenovirus B*	dsDNA	NC_004001	34794
Human adenovirus C*	dsDNA	NC_001405	35937
Human adenovirus D*	dsDNA	NC_002067	35100
Human adenovirus E*	dsDNA	NC_003266	35994
Human adenovirus type 40*	dsDNA	NC_001454	34214
Human adenovirus type 41*	dsDNA	DQ315364	34189
Norwalk virus*	ssRNA positive no DNA state	NC_001959	7654
Sapovirus*	ssRNA positive no DNA stage	NC_010624	7458
Human enterovirus A*	ssRNA positive no DNA stage	NC_001612	7413
Human enterovirus B*	ssRNA positive no DNA stage	NC_001472	7389
Human enterovirus C*	ssRNA positive no DNA stage	NC_001428	7401

Table 5.1 continued

Human enterovirus D*	ssRNA positive no DNA stage	NC_001430	7390
Human enterovirus E*	ssRNA positive no DNA stage	NC_003988	7374
Poliovirus*	ssRNA positive no DNA stage	NC_002058	7440
rotavirus A†	dsRNA virus	AB077766 (VP4 gene)	2359
		AB071404 (VP7 gene)	1062
rotavirus B†	dsRNA virus	AY539857 (VP4 gene)	2306
		AY539856 (VP7 gene)	814
rotavirus C†	dsRNA virus	AB008670 (VP4 gene)	2283
		AB008671 (VP7gene)	1063
Coronavirus*	ssRNA positive no DNA stage	NC_002645	27317
cytomegalovirus (HH5)*	dsDNA virus	NC_006273	235645
Torovirus‡	ssRNA positive no DNA stage	AF159585 (Hemagglutinin esterase gene)	1251
		AF024539 (nucleocapsid gene)	219

Table 5.1 continued

Picobirnavirus[§]	dsRNA virus	AF246940 (RNA dependent RNA polymerase)	1674
		AF246941 (segment 1 gene)	1572
Astroviruses[*]	ssRNA	NC_001943	6813
JC polyomavirus[*]	ds DNA	NC_001699	5130
BK polyomavirus[*]	ds DNA	NC_001538	5153

[*] Complete genome

[†] VP4 and VP7

[‡] Hemagglutinin esterase and nucleocapsid protein mRNA

[§] RNA dependent RNA polymerase and segment 1 gene

5.3.3 Preparation of Samples for Hybridization

Viral RNA was labeled with fluorescent dyes by a semi-random primed labeling with Sensiscript III reverse transcriptase as described by Wang et al. (Wang et al., 2003). Half a microgram (0.5 μg) of viral nucleic acid was used as a template for the generation of cDNA with a discrete 5' terminal consisting of the sequence (5'-GTTTCCCAGTCACGATC-3') using the semi-random primer A: (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3'). Next primer B: (5'-GTTTCCCAGTCACGATC-3') and Qiagen Hotstart Taq polymerase was used to amplify the generated cDNA and label it with amino-allyl dUTP for 40 cycles using the following profile: 30 seconds at 94°C, 30 seconds at 40°C, 30 seconds at 50°C, 60 seconds at 72°C. To label viral DNA, 0.5 micrograms of virus DNA was first digested with 1U of DPNII restriction endonuclease at 37 °C for 1 hour. The digested DNA was incubated at 37 °C for 2 hours with Klenow enzyme and Primer A to generate complementary DNA with a discrete 5' terminal. Next primer B and Qiagen Hotstart Taq polymerase was used to amplify the generated cDNA and label it with amino-allyl dUTP for 40 cycles using the following profile: 30 seconds at 94°C, 30 seconds at 40°C, 30 seconds at 50°C, 60 seconds at 72°C for 40 cycles.

Labeled DNA and RNA were coupled separately with Cyanine dye 3 and Cyanine dye 5. This reaction was carried out in the absence of light and using 1 molar sodium bicarbonate (pH 9.5) as a coupling buffer with a 1-hour incubation. Following coupling, uncoupled dye was removed using the QIAgen PCR purification kit and the labeled virus DNA and RNA were dessicated in a DNA 120 SpeedVac (Thermo Fisher Scientific, MA,

USA) for 1.5 hours. The sample preparation, labeling and hybridization procedure is outlined in Figure 5.1.

5.3.3 Hybridization of Samples

Microarray hybridization was performed as described previously (Wick et al., 2006). The microarrays were hybridized and washed in a M-2 microfluidic station (Invitrogen, Carlsbad, CA, formerly Xeotron Corporation, Houston, TX) using a flow rate of 400 microliters / minute. The hybridization buffer was 6x· SSPE, 25% formamide, 0.4% Triton X-100. Chips were pre-hybridized with 6x· SSPE, 0.2% Triton X-100 and then with hybridization buffer for 2 min each.

Labeled targets were resuspended in 50 microliters hybridization buffer, denatured at 95°C for 3 minutes, cooled on ice for 1 minute, filtered through a 0.22 micrometer Costar spin filter and then hybridized to the chip for 14–15 hours at 20°C.

The chip was scanned with a GenePix 4000B laser scanner (Axon Instruments, Union City, CA). All solutions were filtered through a 0.22 micrometer filter to prevent clogging of the microfluidic channels. The high stringency wash buffer was degassed under vacuum. All arrays were imaged with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) and GenePix Pro software.

5.3.5 Data Analysis Using DetectiV

The DetectiV software package was used to visualize, normalize and test the significance of the microarray hybridization data (Watson et al., 2007). DetectiV was used to generate bar plots of the viral microarray signals following normalization against the median signal values for each sample. DetectiV was also used to carry out statistical t-test comparisons of the hybridization signal values between different virus groups in order to determine which groups of virus target had statistically significant probe signals.

5.3.6 Polymerase chain reaction (PCR) detection of viral targets

Cell culture extracts were also evaluated with PCR for key viruses. PCR was performed using the QIAgen OneStep Reverse transcription kit for RNA viruses and the QIAgen Hotstar Taq kit for DNA viruses. The OneStep Reverse Transcription reaction was carried out as follows: 2 microliters of OneStep RT-PCR enzyme mix, 1.5 millimolar $MgCl_2$, 1× PCR buffer, 1× Q solution, 0.5 micromolar of each primer, 0.5 millimolar of each dNTP and 0.5 microgram template. Thermocycler settings for the reverse transcription PCR were as follows: 50°C, 30 minutes first strand synthesis, 95 °C, 15 minutes initial denaturation to activate the Hotstart Taq polymerase (Qiagen; Valencia, CA), 95°C, 0.5 minutes denaturation, 57 °C, 0.5 minutes annealing and 72°C, 0.5 minutes elongation for 35 cycles followed by a final elongation step of 72°C for 5 minutes. The PCR reaction was carried out as follows: 1 unit of Hotstart Taq polymerase, 1.5 millimolar $MgCl_2$, 1× PCR buffer, 1× Q solution, 0.5 micromolar of each primer, 0.5

millimolar of each dNTP and 0.5 microgram of template. Thermocycler settings for the PCR reaction were as follows: 95 °C, 15 minutes initial denaturation to activate the Hotstart Taq polymerase (Qiagen; Valencia, CA), 95°C, 0.5 minutes denaturation, 57°C, 0.5 minutes annealing and 72°C, 0.5 minutes elongation for 35 cycles followed by a final elongation step of 72°C for 5 minutes. PCR primers used are reported in table 5.2.

Table 5.2. List of primers used for the polymerase chain reaction PCR detection and sequencing for each viral target

Virus	Primers	Estimated Product Size	Source
Hepatitis E virus	HE364: 5′-CTG GGM YTG GTC DCG CCA AG-3′ HE361: 5′-GCR GTG GTT TCT GGG GTG AC-3′	164 bp	(Inoue et al., 2006)
Human adenovirus A	hexAA1885 : 5'-GCCGCAGTGGTCTTACATGCACATC-3' hexAA1913 : 5'-CAGCACGCCGCGGATGTCAAAGT-3' hexAA1893: 5'-GCCACCGAGACGTACTTCAGCCTG-3' hexAA1905: 5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'	301 bp 143 bp	(Chapron et al., 2000)
Human adenovirus B			
Human adenovirus C			
Human adenovirus D			
Human adenovirus E			
Human adenovirus type 40			
Human adenovirus type 41			
Norwalk virus	GIF: TGTCACGATCTCATCATCACC GIR: GTGAACAGC/TATAAAT/CCACT/CGG GIIF: TGTCACGATCTCATCATCACC GIIR: TGGAATTCCATCGCCCACTGG	123 bp 123 bp	(Borchardt et al., 2003)
Sapovirus	F22: SMWAWTAGTGTTTGARATG R2: GWGGGRTCAACMCCWGGTGG	420 bp	(Okada et al., 2006)

Table 5.2 continued

Human enterovirus A	RT: 5'-ATTGTCACCATAAGCAGCCA-3' PCR: 5'-CGGTACCTTTGTACGCCTGT-3' Nested: 5'-TCCGGCCCCTGAATGCGGCTA-3' Nested: 5'-GAAACACGGACACCCAAAGTA-3'	534 bp 138 bp	(Chapron et al., 2000)
Human enterovirus B			
Human enterovirus C			
Human enterovirus D			
Human enterovirus E			
Poliovirus			
Hepatitis A virus			
rotavirus A	TTGCCACCAATTCAGAATAC ATTTCGGACCATTTATAACC	211 bp	(Borchardt et al., 2003)
rotavirus B			
rotavirus C			
Coronavirus	Cor-FW : 5'-ACWCARHTVAAYYTNAARTAYGC-3' Cor-RV : 5'-TCRCAYTTDGGRTARTCCCA-3'	251 bp	(Moes et al., 2005)
cytomegalovirus (HH5)	IE: GCTGCGGCATAGAATCAAGGAGCA IE: GGTTGGTGGTCTTAGGGAAGGCTGAG	393 bp	(Caballero et al., 1997)

Table 5.2 continued

Torovirus	TRVF: CACCACGTAATCAGTATAG TRVR: GTACGACACACAACATC	160 bp	This Study
Astroviruses	RT: 5'-GTAAGATTCCCAGATTGGT-3' PCR: 5'-CCTGCCCCGAGAACAACCAAG-3' A1: 5'-CCTTGCCCCGAGCCAGAA-3' A2: 5'-TATTCACAAACTTATGGCAA-3'	243 bp 143bp	(Chapron et al., 2000)
JC polyomavirus	JCSR : 5'-TGATTACAGCATTTTTGTCTGCAAC-3' JCSL : 5'-GGAAGTCCTTCTGTTAATTAAATCAG-3'	170 bp	(Bofill-Mas et al., 2001)
BK polyomavirus	BK3 5'-ACTGTAACACCTGCTCTT-3' BK4 5'-AGTAGATTTCCACAGGTTAG-3'	350 bp	(Bofill-Mas et al., 2001)

5.3.7 Sequencing confirmation of viral targets

PCR products were purified using the QIAgen QIAquick PCR purification kit and eluted with molecular grade water. 10 nanograms of purified PCR product and 30 picomoles of each primer were provided to the Michigan State University Research Technology Support Facility for custom sequencing. Sequencing results were reassembled from the forward and reverse sequencing runs by hand and the sequences were used to perform a Basic Local Alignment and Search Tool (BLAST) query (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to determine the closest matches based on sequence homology.

5.4 Results and Discussion

5.4.1 Hybridization with poliovirus LS-C-1, Adenovirus type 40 and 41 Laboratory Strains

The viral microarray was tested with a laboratory strain of poliovirus LS-C-1 grown in African Green Monkey Kidney cells (BGM). Viral RNA was extracted using the QIAgen viral RNA kit and labeled with Cy3 dye. All 30 probes generated for poliovirus hybridized to the cell culture poliovirus samples with zero cross hybridization above a signal to noise ratio cutoff of 2 with non-poliovirus probes. Hybridizations were repeated twice with similar results. Hybridization signal values were deposited in GEO (accession #GSE10566). Hybridization signals were normalized against the median signal intensity as described by Watson et al (2007), $\log_2$ transformed and averaged across the three runs. Log_2-transformed mean intensity signals were used to generate a bar plot shown in figure 5.2a. A "t-test" was carried out to test the null hypothesis that

hybridization signals between viral target groups were not significantly different. Based on the t test, at the 95% confidence level, the poliovirus group was found to have a significantly greater signal than the other viral groups with a p value of 1.74×10^{-28} and an average normalized signal value of 3.00, leading to a rejection of the null hypothesis for poliovirus (Table 5.3).

Adenovirus type 40 strain Dugan (ATCC #VR-931) and Adenovirus type 41 strain Tak (ATCC #VR-930) were extracted directly from the stock vials, labeled with Cy3 and Cy5 respectively and co-hybridized on to the viral microarray. Hybridization signal values were deposited in GEO accession # GSE10569. Log_2-transformed and normalized signals were plotted on a bar graph (Figure 5.2b). Separate "t tests" were carried out for each channel (Cy3 and Cy5) to test the null hypothesis that hybridization signals between viral target groups were not significantly different. Based on the t tests, at the 95% confidence level, the Cy3-labeled adenovirus type 40 was found to generate significant signals with probes designed specifically for adenovirus type 40 (p value = 1.47×10^{-9}, average normalized intensity = 2.776) and also adenovirus type 41 probes (p value=2.10×10^{-5}) but with a lower average normalized signal intensity (average normalized intensity=1.034) . Similarly, the Cy5-labeled adenovirus type 41 was found to generate significant signals with probes designed for adenovirus type 41 (p value = 7.09×10^{-9}, average normalized intensity = 2.690) and also adenovirus type 40 probes (p

value=7.64×10^{-6}) but with a lower average normalized signal intensity (average normalized intensity=0.938).

5.4.2 Using a positive fraction criteria for assigning a probable viral target presence

Based on the fluorescent intensities for each spot, we characterized a probe as being positive when at least 4 out of the 5 copies of that probe showed a signal to noise value greater than 2. When adenovirus type 40 and 41 signals were expressed as a fraction of the maximum possible signal and charted on a histogram together with the next forty highest non-adenovirus type 40 or 41 signals it was observed that only 12 out of the possible 30 probes for each virus were positive by our criteria (Figure 5.3). From this, we concluded that we would expect approximately 12 probes from the target viruses to show positive signals greater than background levels when any given mixed consortia of viruses is hybridized to the array. This theoretical number of 12 probes was used to form the upper limit for determining the positive fraction which is the number of positive probes divided by 12.

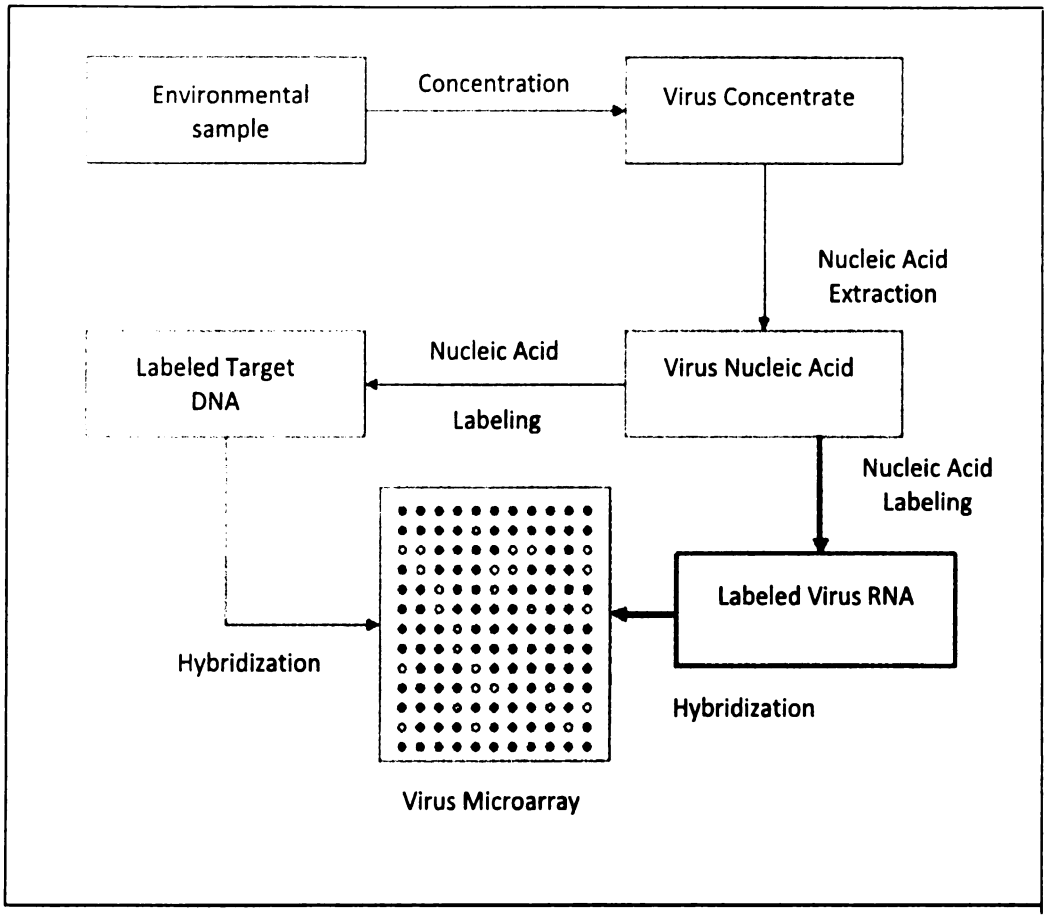


Figure 5.1. Schematic illustrating the steps involved with virus concentration, nucleic acid extraction, labeling and hybridization on the virus microarray resulting in a hybridization pattern

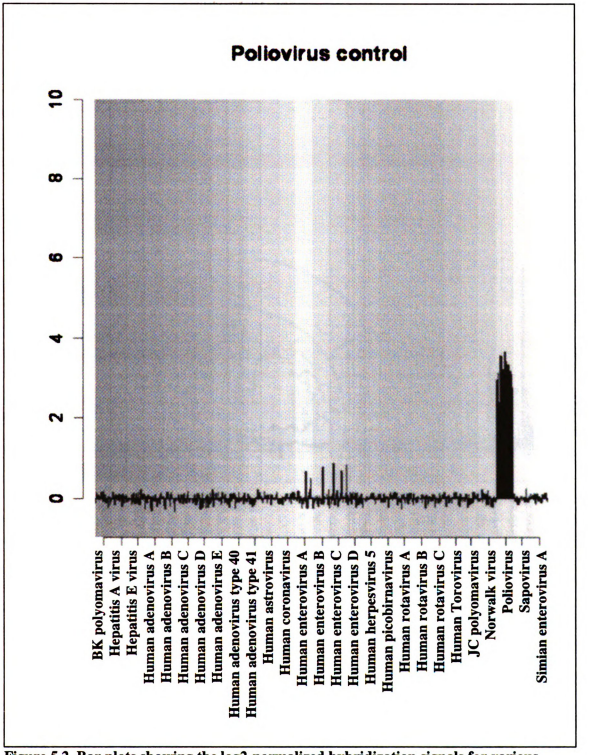
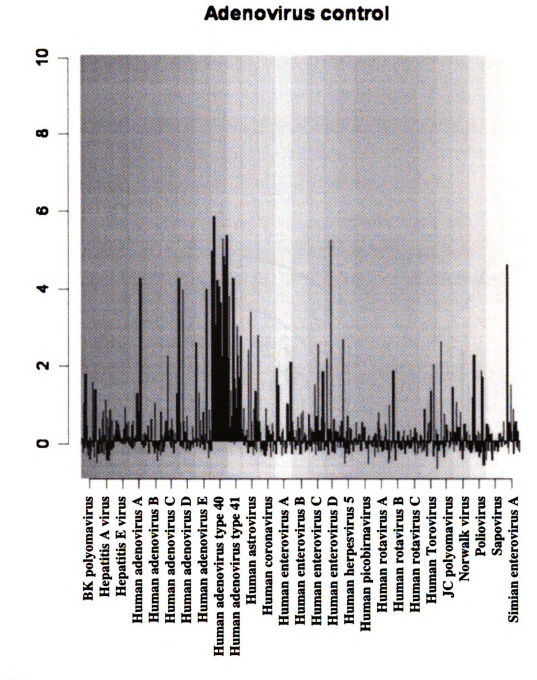
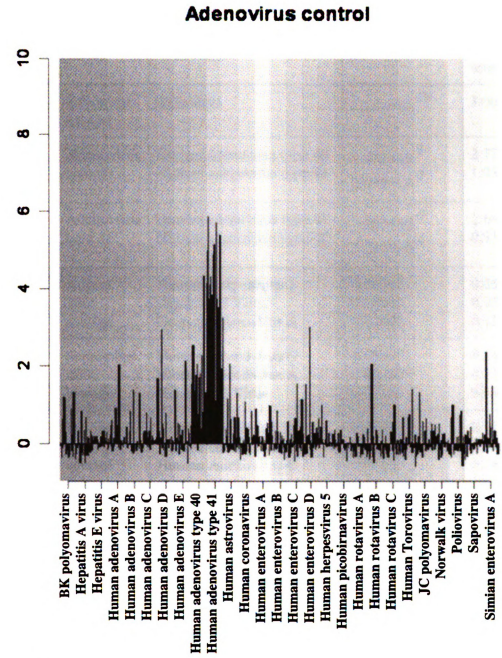


Figure 5.2. Bar plots showing the log2-normalized hybridization signals for various control experiments. (a) Hybridization of poliovirus LS-C-1 RNA extracts labeled with Cy3.



(b)

Figure 5.2 cont'd. (b) Hybridization of Cy3-labeled adenovirus type 40 unto the viral array. Each vertical bar represents a different target virus group.



(c)

Figure 5.2 cont'd. (c) Hybridization of Cy5-labeled adenovirus type 41 unto the viral array. Each vertical bar represents a different target virus group.

Table 5.3. t test results for the hybridization of control viruses and sewage extracted samples to the viral microarray showing the most significant results with p values less than 0.01 and their corresponding $\log_2$ mean intensity

Sample type	Sample	Top targets	p values	$\log_2$ mean intensity
Control	Poliovirus LS-C-1	Poliovirus	1.741×10^{-28}	3.0056
Control	Adenovirus type 40	Human adenovirus type 40 Human adenovirus type 41	1.472×10^{-9} 2.099×10^{-5}	2.7757 1.0340
Control	Adenovirus type 41	Human adenovirus type 41 Human adenovirus type 40	7.087×10^{-9} 7.641×10^{-6}	2.6902 0.9376
Sample	August 2006 sewage	Human Herpesvirus 5 Hepatitis E Virus Human Enterovirus A	0.00291 0.00466 0.01002	0.05398 0.07704 0.12030
Sample	September 2006 sewage	Human Adenovirus C Human Adenovirus A Hepatitis E Virus	0.00257 0.00310 0.01361	0.14788 0.12599 0.08063
Sample	October 2006 sewage	Human enterovirus D Human enterovirus E Human Adenovirus B	0.00411 0.00563 0.01652	0.21707 0.24263 0.18649
Sample	November 2006 sewage	Human enterovirus E Human enterovirus A Human Astrovirus	0.00558 0.01134 0.01587	0.18725 0.22106 0.27328
Sample	December 2006 sewage	Human enterovirus A Human Astrovirus Human enterovirus E Human Adenovirus type 41	0.00114 0.00121 0.00161 0.00253	1.15508 1.09681 0.97785 0.61050
Sample	January 2007 sewage	Human Adenovirus type 41 Human Astrovirus Human Enterovirus A Human Enterovirus E Norwalk virus Human Enterovirus D Human Enterovirus B Sapovirus Human Adenovirus B	0.00029 0.00032 0.00044 0.00049 0.00457 0.00516 0.00585 0.00615 0.00761	1.24453 1.48500 1.33010 1.19024 0.89360 0.84998 0.75816 0.70991 0.98407

Table 5.3 Continued

Sample	February 2007 sewage	Human adenovirus type 41 Human astrovirus Human enterovirus B Human enterovirus A	0.00033 0.00095 0.00417 0.00753	1.10309 1.29553 0.75738 0.78814
Sample	March 2007 sewage	Human Herpesvirus 5 Human Enterovirus B Hepatitis E Virus	2.290×10^{-05} 0.00081 0.00434	0.41377 0.22016 0.11590
Sample	April 2007 sewage	Sapovirus Human Herpesvirus 5 Hepatitis E Virus	7.36×10^{-07} 2.15×10^{-05} 3.86×10^{-03}	0.25954 0.41552 0.11773
Sample	May 2007 sewage	Sapovirus Human Herpesvirus 5 Human Enterovirus B	7.46×10^{-07} 2.13×10^{-05} 1.11×10^{-03}	0.25990 0.41574 0.21538
Sample	June 2007 sewage	Sapovirus Human Herpesvirus 5 Human Enterovirus A	7.46×10^{-07} 2.25×10^{-05} 6.75×10^{-05}	0.25912 0.41541 0.26078
Sample	July 2007 sewage	Sapovirus Human Herpesvirus 5 Human Enterovirus A	7.52×10^{-07} 2.85×10^{-05} 6.75×10^{-05}	0.25938 0.41257 0.26011
Sample	August 2007 sewage	Sapovirus Human Herpesvirus 5 Human Enterovirus B	7.65×10^{-07} 2.67×10^{-05} 1.05×10^{-03}	0.25731 0.32781 0.21546

5.4 3 Hybridization of Sewage Extracts

Cy3 (DNA) and Cy5-labeled (RNA) nucleic acids derived from sewage samples collected between August 2006 and August 2007 were hybridized on to the viral microarray. Hybridization signals were deposited at the Gene Expression Omnibus (GEO) as accession #GSE11195. Based on a positive fraction cut off of at least 0.33 representing 4 out of the arbitrary upper limit of 12 positive probes, the potential virus detected by the microarray are listed in table 5.4. Using the software package DetectiV, bar plots, illustrating the hybridization intensities for the various sewage samples were created and are shown in figure 5.4. It was observed that much higher normalized signal intensities were obtained during the winter months compared to the summer months – average signal intensities for the top five most significant virus groups were approximately 1 in December, January and February while in August, September, April, May, June and July they were approximately 0.1, except for a few strong positives that were observed (Table 5.3). In the August 2006 sample, the most significant t value was found to belong to the Human Herpesvirus group 5 (Table 5.3). However, the $\log_2$ mean signal intensity for that group of viruses was only 0.05398, thus it should not be considered as the most likely virus in that sample. As discussed by Watson et al (Watson et al., 2007), it is more likely a Human enterovirus group A virus (p value = 0.01002, log mean signal intensity = 0.1203).

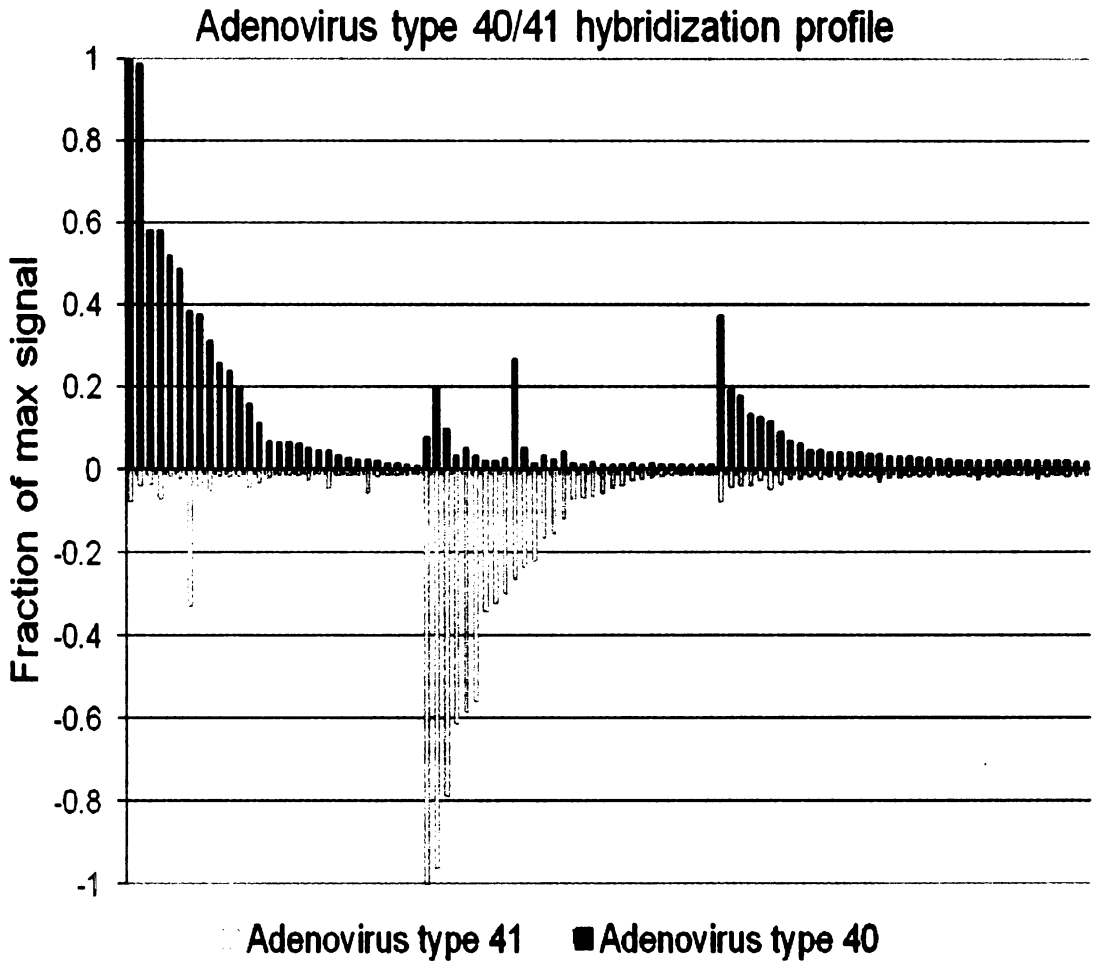


Figure 5.3 Adenovirus type 40 and 41 hybridization results. Adenovirus type 40 signals are shown above the x-axis line and adenovirus type 41 signals are shown below the x-axis line.

Table 5.4. Comparison of positive signal fractions, software analysis, group specific PCR results and sequencing data for the identification of viruses within a sample

Sample	Potential targets via positive fraction analysis	Potential targets via software analysis	PCR results	Sequenced identities
August 2006			+ +	Human adenovirus type 41 Human astrovirus type 7
September 2006			+	Human astrovirus type 7
October 2006			+ +	Human adenovirus type 32 Human enterovirus A-2 plaque
November 2006	Human coronavirus		-	
			+	Human astrovirus type 7
December 2006	Human astrovirus	Human astrovirus	+	Human astrovirus type 7
	Human coronavirus		-	
	Human enterovirus A	Human enterovirus A	+	Human coxsackie A10
	Human enterovirus E	Human enterovirus E		
	Norovirus		+	Norovirus isolate
	Human rotavirus C		+	Human rotavirus C isolate
	Human Adenovirus B		+	Human adenovirus type 7
		Human adenovirus type 41		
			+	BK polyomavirus strain AS

Table 5.4 Continued

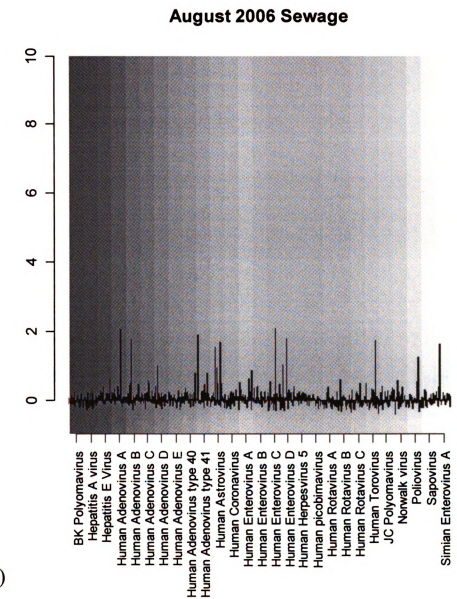
December 2006			+	Hepatitis E virus
			+ (human torovirus)	No homology
January 2007	Human astrovirus	Human astrovirus	+	Human astrovirus type 7
	Human coronavirus		-	
	Human enterovirus A	Human enterovirus A	+	Human enterovirus A-2 plaque
	Human enterovirus B			
	Human enterovirus C			
	Human enterovirus D	Human enterovirus D		
	Human enterovirus E	Human enterovirus E		
	Hepatitis E virus		+	Hepatitis E virus
	Norovirus	Norovirus	+	Norovirus isolate
	Sapovirus	Sapovirus		
	Human Rotavirus B		+	Human rotavirus B
	Human Rotavirus C			

Table 5.4 Continued

January 2007	Human Adenovirus A		+	Human adenovirus type 41
	Human Adenovirus B	Human adenovirus B		
	Human Adenovirus D			
	Human Adenovirus type 41	Human adenovirus type 41		
	BK polyomavirus		+	BK polyomavirus strain AS
			+ (human torovirus)	No homology
February 2007	Human astrovirus	Human astrovirus	+	Human astrovirus type 7
	Human coronavirus		-	
	Human enterovirus A	Human enterovirus A	+	Human coxsackie A10
		Human enterovirus B		
	Human adenovirus B		+	Human adenovirus type 41
	Human adenovirus type 41	Human adenovirus type 41		
	BK polyomavirus		+	BK polyomavirus strain AS
March 2007			+	Human coxsackie virus A20

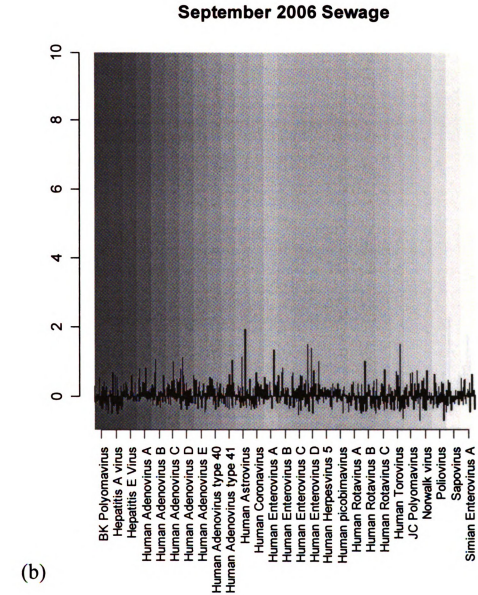
Table 5.4 Continued

April 2007			+	Human coxsackie virus A 20
May 2007				
June 2007			+ +	Human astrovirus type 7 Human adenovirus type 41
July 2007				
August 2007			+	Human coxsackie virus A16



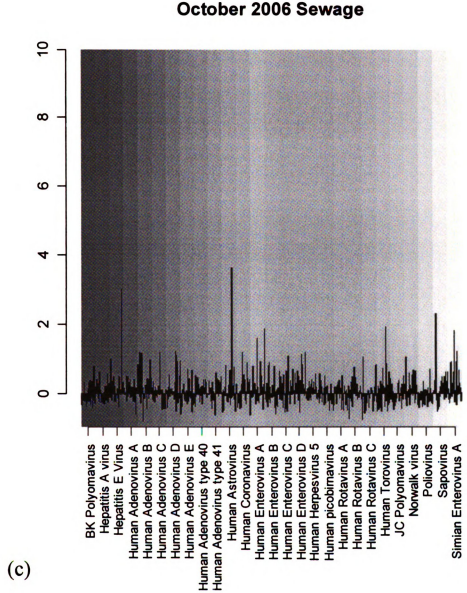
(a)

Figure 5.4. Bar plot showing log_2-normalized hybridization signals for the different target virus groups when Cy3-labeled DNA and Cy5-label RNA extracted from sewage is hybridized unto the array. (a) August 2006 sample. Each vertical bar represents a different target virus group.



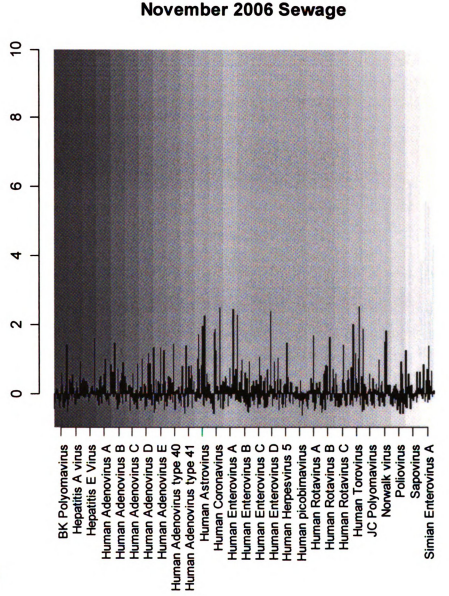
(b)

Figure 5.4 (Continued). (b) September 2006 sample. Each vertical bar represents a different target virus group.



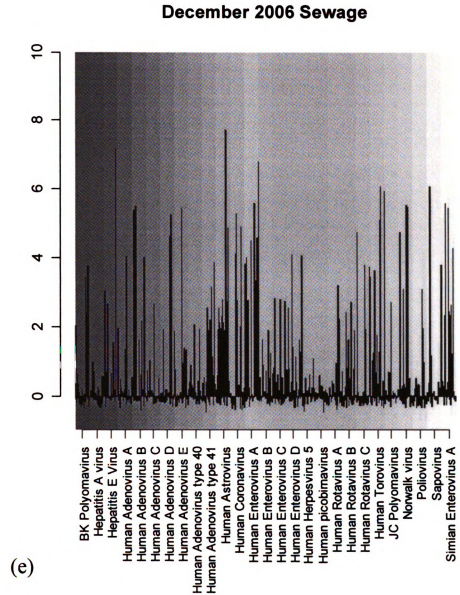
(c)

Figure 5.4 (Continued). (c) October 2006 sample. Each vertical bar represents a different target virus group.



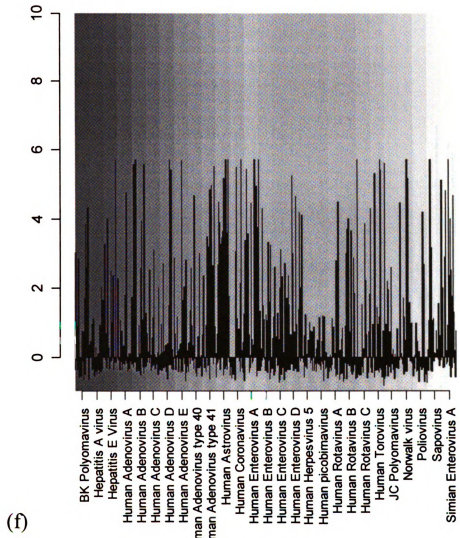
(d)

Figure 5.4 (Continued). (d) November 2006 sample. Each vertical bar represents a different target virus group.



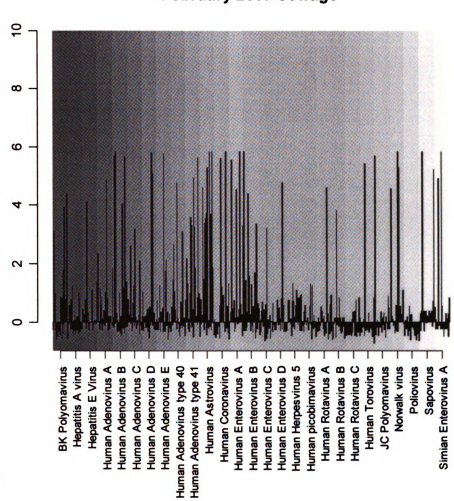
(e)

Figure 5.4 (Continued). (e) December 2006 sample. Each vertical bar represents a different target virus group.



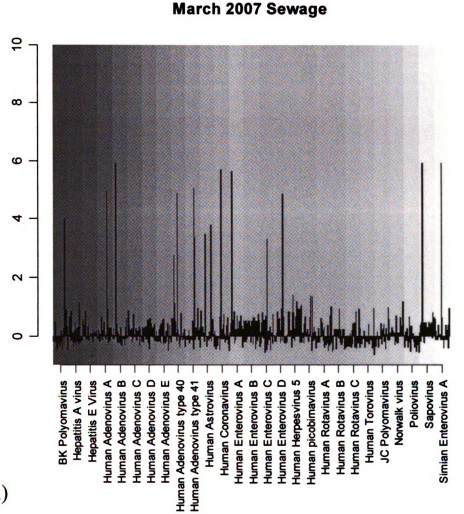
(f)

Figure 5.4 (Continued). (f) January 2007 sample. Each vertical bar represents a different target virus group.



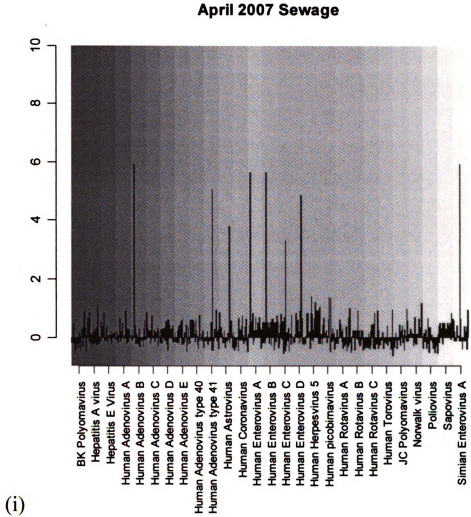
(g)

Figure 5.4 (Continued). (g) February 2007 sample. Each vertical bar represents a different target virus group.



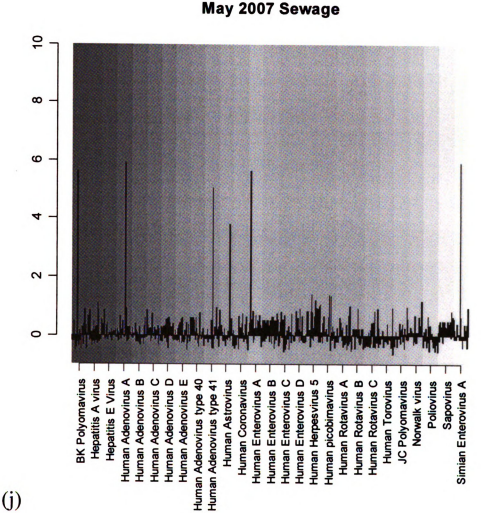
(h)

Figure 5.4 (Continued). (h) March 2007 sample. Each vertical bar represents a different target virus group.



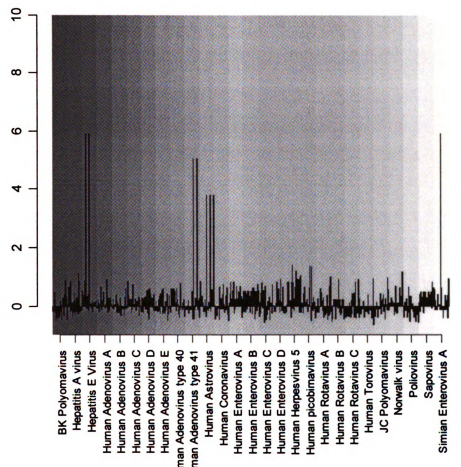
(i)

Figure 5.4 (Continued). (i) April 2007 sample. Each vertical bar represents a different target virus group.



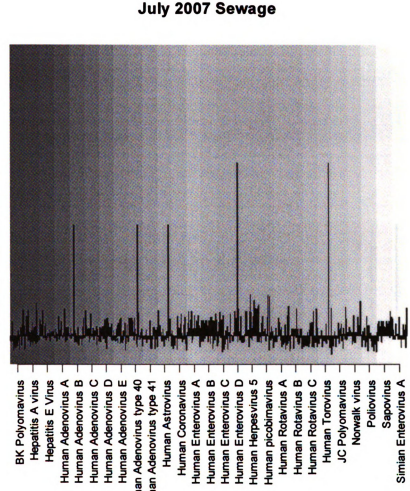
(j)

Figure 5.4 (Continued). (j) May 2007 sample. Each vertical bar represents a different target virus group.



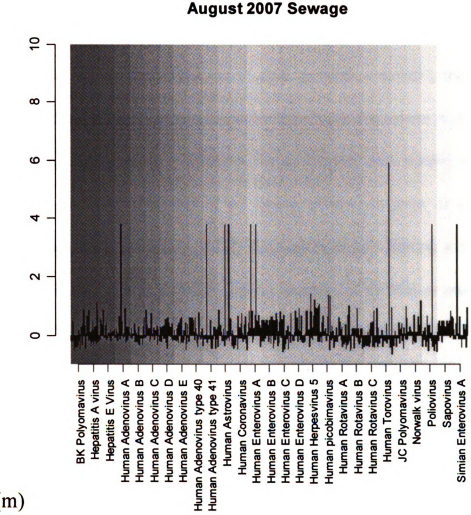
(k)

Figure 5.4 (Continued). (k) June 2007 sample. Each vertical bar represents a different target virus group.



(l)

Figure 5.4 (Continued). (l) July 2007 sample. Each vertical bar represents a different target virus group.



(m)

Figure 5.4 (Continued). (m) August 2007 sample. Each vertical bar represents a different target virus group.

For the other samples, identification of the putative pathogen(s) present was more evident. In the September 2006 sample, the most likely viruses to have been present were a member of the Human Adenovirus C group (p value = 0.00257, $\log_2$ mean signal intensity = 0.14788) followed by a member of the Human Adenovirus A group (p value = 0.0031, $\log_2$ mean intensity = 0.12599). For the October 2006 sample, the most likely viruses present were a member of the Human enterovirus group D group (p value 0.00411, $\log_2$ mean intensity = 0.217) followed by viruses bearing a homology with Simian enterovirus group A (which includes members of the Human enterovirus group E). In the November 2006 sample, the most likely viruses present were a member of the Human enterovirus group E viruses (represented on the array by Simian enterovirus group A) with Human enterovirus group A and astroviruses being potential agents as well (p values = 0.00113, 0.0159 and $\log_2$ mean intensity = 0.221 and 0.273 respectively). Multiple viruses were found to be present in the sample from December 2006 – Human enterovirus group A, Human astroviruses, Human enterovirus group E (represented on the array by Simian enterovirus group A sequences) Human adenovirus type 41 and Human enterovirus group E.

Similarly, in the January 2007 sample, the putative viruses present as indicated by DetectiV were Human Adenovirus type 41, Human Astrovirus, Human Enterovirus group A, Human enterovirus group E (represented on the array by Simian Enterovirus group A),

Human Herpesvirus type 5, Norwalk virus, Human Enterovirus D, Human Enterovirus B, Sapovirus, and Human Adenovirus B.

5.4.4 Comparison of probable viral target detection using positive fraction analyses and software analyses with PCR and sequencing confirmation

Based on the t test results, the most probable virus present in each sample, as determined by the DetectiV software package and utilizing a p value cut off of less than 0.01 and a $\log_2$ mean intensity value of at least 0.5, are listed in table 5.4 and compared against the probable identification obtained through positive fraction analyses. In general, DetectiV was more conservative in proposing the presence of a viral target compared to positive fraction analyses. Nevertheless there is agreement between the two methods as shown in table 5.4, particularly for adenovirus and astrovirus targets. Polymerase chain reaction primers and amplified products appear to be more sensitive, detecting viruses that the microarray could not pick up in several months and identified 13 different types of viruses with Adenovirus type 41 and Astroviruses type 7, identified most frequently (in 4 and 7 months, respectively, table 5.4). The enteroviruses (including enterovirus A-2, Coxsackieviruses A10, A20 and A16) were identified during 7 of the 13 months surveyed.

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., 2006). To date, only a few examples exist for the application of microarray technology on environmental samples and even fewer as a pathogen detection tool (Call et al., 2003;

Zhou, 2003; Sergeev et al., 2004; Maynard et al., 2005; Lee et al., 2006; Quinones et al., 2007; Miller et al., 2008). One drawback of the use of microarrays for pathogen detection is the lengthy hybridization time, often more than 12 hours. In order to reduce the time to result, several modifications have been suggested. Examples of modified microarray technology include Barlaan et al. who combined electric field-driven migration of nucleic acid targets to specific test sites with a detection microarray. Using this electronic microarray they are able to achieve hybridization results in minutes as opposed to the usual hours. Barlaan et al. applied this technology to the detection of harmful algal blooms in coastal and microcosm environments (Barlaan et al., 2007). Ahn et al. likewise examine harmful algal blooms with a modified microarray that is visualized through fibre optic bundles, allowing the hybridization assay to be carried out in-situ (Ahn et al., 2006).

5.5 Conclusion

Based on the results obtained, we conclude that there is a great diversity in the types of human enteric viruses circulating among a given community. Generally, the most frequently detected family of viruses year round from sewage were the group A Human enteroviruses from the picornaviridae family. The second most frequently detected virus family were the Human adenoviruses from the adenoviridae family. Human caliciviruses as represented on the array by the Norovirus, human astrovirus and sapoviruses were detected during the winter months, indicating a possible seasonality to these groups of viruses as has been previously reported (Mounts et al., 2000). From our results it appears that human pathogenic viruses were present among community during the periods of sampling at levels high enough to be detected by hybridization unto an

array even though no outbreaks of these diseases were reported among the community. This corroborates research indicating that viral gastroenteritis diseases are frequently under-reported (Majowicz et al., 2005; Day and Sutton, 2007). The presence of viruses in the sewage without reports of outbreaks could also represent asymptomatic shedding of viruses (Gallimore et al., 2004; Mendez-Toss et al., 2004; Nwachuku and Gerba, 2006; Monica et al., 2007).

To our knowledge, this study demonstrates the first use of a microarray to characterize human pathogenic viruses in the environment. Target detection microarrays are increasingly being accepted as a viable tool for environmental monitoring. Several hurdles still remain that prevent the widespread use of arrays in environmental application. The first has to do with the presence of inhibitors in the sample which can lower labeling efficiency. Second, is the need for extensive validation of the signals observed via alternative means. Third is the lack of software to analyze, visualize, normalize and carry out significance testing on pathogen array data. Also, the cost associated with designing and implementing a microarray detection system remains too high for routine use. Despite these drawbacks, the potential applications for these microarrays are tremendous and include multiple pathogen detection, community health screening and monitoring, bioterrorism surveillance, monitoring the expression of key metabolic genes, and pathogen discovery. The ability to screen community health via excretion of viral pathogens in urine and feces, means that we could improve our understanding of exposure, disease and ultimately prevention strategies. In addition, we

are working on reducing the cost and hybridization time by an order of magnitude as well as eliminating the need for a scanner for yes or no type of diagnosis uses.

CHAPTER 6 TESTING THE SENSITIVITY OF THE MICROARRAY WITH POLIOVIRUS SPIKED INTO SEWAGE

6.1 Introduction

In order to determine the level of sensitivity of detection for the virus microarray, a series of hybridizations were carried out to determine the signal response when increasing levels of poliovirus LS-C-1 was spiked into the cell culture supernatant of flasks that had developed cytopathic effects after being exposed to raw sewage concentrates. A range of concentrations of poliovirus LS-C-1 between 12 and 12,000 plaque forming units was chosen to express levels of virus reflective of those thought to be present in sewage and the environment. Poliovirus was chosen for its ease of culture as well as enumeration. In addition, vaccination using the live attenuated strain of poliovirus had been discontinued since 2000 and a previous year long survey of the same plant's sewage never indicated the presence of poliovirus above the detection criteria.

6.2 Literature Survey

6.2.1 Levels of Human Viruses in Water

Several studies have been conducted to determine the concentration of human viruses in various environmental matrices. Concentrations of human viruses are highest in raw sewage, approximately 10 to 100 times lower in wastewater effluent and hundreds to tens of thousands of times lower in receiving waters. A study by Albinana-Gimenez et

al (2006) measured levels of human polyomavirus, adenovirus and hepatitis E virus and found that the average concentration of JC polyomavirus in a Spanish river was 26 genome copies per liter and that human adenoviruses have an average concentration of 400 genome copies per liter using quantitative polymerase chain reaction detection. Hepatitis E virus was frequently detected at low levels in urban sewage, biosolids and sewage containing swine feces but was not observed in the river water samples (Albinana-Gimenez et al., 2006).

Another study of treated wastewater by Gantzer et al. (1998) found that the levels of infectious enterovirus ranged from <1 most probable number of cytopathic units (MPNCU) per liter to 4 MPNCU / liter. The investigators also observed that the percentages of samples testing positive for the enterovirus genome were significantly higher than those for infectious enteroviruses and attributed this finding to either the presence of noninfectious enteroviruses or to the presence of infectious enteroviruses that do not multiply in BGM cell cultures (Gantzer et al., 1998).

Two studies by Haramoto et al. (2007, 2008) which looked at the concentrations of human adenoviruses and sapoviruses in the aquatic environment found that the enteric serotypes of HuAdVs were detected at the concentration of 7.3 -1500 PCR-detection units (PDU) per milliliter in raw sewage, 0.00060 - 4.1 PDU per milliliter in secondary-treated sewage before chlorination, 0.0018-7.0 PDU per milliliter in river water, and 0.032-6.1 PDU per milliliter in seawater (Haramoto et al., 2007). On the other hand, the concentration of sapoviruses in influent ranged from 2.8×10^3 to 1.3×10^5 copies per

liter, showing a higher value in winter. In all, seven (58%) of 12 effluent samples tested were positive for sapoviruses, as were 23 (64%) of 36 river water samples collected from three sites along the Tamagawa River (Haramoto et al., 2008).

One study by Jiang et al. (2001) which looked at the levels of human adenoviruses in coastal waters detected similar levels of virus as those determined by Haramoto et al. (2007). Jiang et al.(2001) detected 880 to 7,500 most probable numbers of adenovirus genomes per liter of water. The investigators concluded that the prevalence of adenoviruses made it a useful indicator of human viral fecal pollution in surface and environmental waters.

6.2.2 Sensitivity of Various Virus Detection Methods

Several papers have been published looking at the method sensitivity of various PCR-based virus detection methods. A detection sensitivity of 0.04 PFU has been reported for hepatitis A virus using immunomagnetic capture reverse transcription polymerase chain reaction in both spiked finished water and environmental samples (Jothikumar et al., 1998). In comparison, the detection sensitivity using conventional reverse transcription polymerase chain reaction was found to be ten times less sensitive at 0.4 PFU (Jothikumar et al., 1998). Another studying using reverse transcription polymerase chain reaction reported that poliovirus could be detected even in the presence of 0.5 milligrams of humic acid or 5.0 milligrams of fulvic at the detection limit of 0.06 plaque forming units (Ijzerman et al., 1997). This study however was carried out using spiked poliovirus in sodium phosphate buffer and might not be indicative of the detection

sensitivity in environmental matrices. A study by Green and Lewis (Green and Lewis, 1995) reported that the sensitivity of detection of enteroviruses in wastewater was between 0.02 and 0.2 plaque forming units per sample. Wyn-Jones et al. found that detection using the polymerase chain reaction gave comparable results to cell culture and in a much shorter time period. Their detection limit was reported to be 5 plaque forming units (pfu)/sample for enteroviruses in river and marine waters (Wyn-Jones et al., 1995). Schwab et al. reported a detection limit of 2 pfu / sample using immunoaffinity concentration and reverse transcription for enteroviruses in fecal-contaminated surface waters (Schwab et al., 1996). Using dot blot hybridization, Pinto et al reported a lower sensitivity of detection compared to polymerase chain reaction-based detection methods. Their limit of detection for astroviruses in environmental water was determined to be 3 pfu per sample (Pinto et al., 1996).

6.3 Materials and Methods

6.3.1 Poliovirus Plaque Assay

Agar overlay plaque assays were carried out to determine the titres of poliovirus for use in the spiking assay to test the sensitivity of the microarray. African Green Monkey (BGM) cells were grown to 80-90% confluence, washed and then exposed to serial dilutions of stock poliovirus LS-C-1. Diluted viruses were allowed contact with the cells for 1 hour with occasional rocking to prevent cells from drying out. Excess diluent was decanted. 10 milliliters of virus agar overlay solution was added to each 25 square centimeter flask and allowed to set. Virus agar overlay solution consists of 1x MEM, 100 U/ milliliter of Penicillin, 100 microgram/ milliliter of Streptomycin and 250

nanogram/milliliter of Fungizone, 0.003% neutral red, 1% sodium bicarbonate, 1.1 % w/v agarose. Cells were incubated at 37 °C and examined daily for the presence of plaques. Flasks with plaque counts of between 10 and 100 were averaged and the concentration of poliovirus LS-C-1 in stock was determined in plaque forming units (pfu) per milliliter.

6.3.2 Wastewater Sample Collection and Cell Culture

A 6 liter samples of untreated sewage was collected in January 2008 from the East Lansing Wastewater Treatment plant. The sample was brought back from the laboratory and 15% buffered beef extract (Difco Inc) was added to give a final concentration of 1.5% beef extract and 0.05 molar glycine. The solution was stirred for 30 minutes before 2.5 molar $FeCl_3$ was added to a final concentration of 2.5 millimolar. The pH of the solution was lowered to 3.5 and the solution was stirred a further 30 minutes. Viruses were pelleted by centrifugation at 2,500 x g for 15 minutes. The viral pellet was resuspended in 90 milliliters of 0.15 molar sodium phosphate dibasic (pH 7.0) solution by agitation on an orbital shaker set to 160 revolutions per minute. Once the pellet was dissolved, the pH of the sodium phosphate was raised to between 9.0 and 9.5 and placed on the orbital shaker for a further 10 minutes. Solid particles were pelleted by centrifugation at 10000 x g (Beckman model J2-HC). The supernatant was collected and filtered through a 0.22 micrometer syringe filter and supplemented with 100 U/ milliliter of Penicillin, 100 microgram/ milliliter of Streptomycin and 250 nanogram/milliliter of Fungizone. The pH of the virus concentrate was neutralized to 7.0 and frozen at –80 °C until placed on cell culture.

Twelve milliliters of virus concentrate was used to infect BGM cells grown to approximately 80 – 90% confluence. Virus concentrate was allowed 120 minutes of contact with the BGM cells before being discarded. Cells were incubated at 37 °C until development of cytopathic effects were observed. Infected cells were harvested by mechanical lifting using a sterile cell scraper. All cells and free viruses in the media were concentrated by centrifugation through an Amicon Ultra 100K centrifugation column (Millipore, Billerica, MA).

Virus nucleic acid was extracted using the Qiagen viral RNA mini kit following the manufacturer's instructions. in the same manner as described above for the ATCC strains of viruses. Briefly, 140 microliters of virus concentrate was added to tubes containing 560 microliters of lysis buffer and incubated at room temperature for 10 min with intermittent mixing. 560 microliters of ethanol was added and the solution mixed before passage through a DNA binding column. Columns were washed with washing solution and eluted with 2 rounds of elution solution using 50 microliters of DNase and RNase free water each time.

6.3.3 Poliovirus spiking

A range of concentrations of poliovirus as determined from the plaque assay described above was used to spike into the cell culture supernatants from the wastewater sample cell culture. Virus particles were recovered and concentrated from the media by centrifugation through an Amicon Ultra 100K centrifugation column (Millipore,

Billerica, MA). Virus nucleic acid was extracted using the QIAamp viral RNA mini kit for both DNA and RNA viruses following the manufacturer's instructions. The QIAamp viral RNA mini kit has previously been demonstrated to be capable of isolating viral DNA as well as viral RNA(Kleines et al., 2003). Briefly, 140 microliters of virus concentrate was added to tubes containing 560 microliters of lysis buffer and incubated at room temperature for 10 min with intermittent mixing. 560 microliters of ethanol was added and the solution mixed before passage through a DNA binding column. Columns were washed with washing solution and eluted with 2 rounds of elution solution using 50 microliters of DNase and Rnase free water each time.

6.3.4 Preparation of Samples for Hybridization

Viral RNA was labeled with fluorescent dyes by a semi-random primed labeling with Sensiscript III reverse transcriptase as described by Wang et al. (Wang et al., 2003). Half a microgram (0.5 μg) of viral nucleic acid was used as a template for the generation of cDNA with a discrete 5' terminal consisting of the sequence (5'-GTTTCCCAGTCACGATC-3') using the semi-random primer A: (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3'). Next primer B: (5'-GTTTCCCAGTCACGATC-3') and Qiagen Hotstart Taq polymerase was used to amplify the generated cDNA and label it with amino-allyl dUTP for 40 cycles using the following profile: 30 seconds at 94°C, 30 seconds at 40°C, 30 seconds at 50°C, 60 seconds at 72°C. To label viral DNA, 0.5 micrograms of virus DNA was first digested with 1U of DPNII restriction endonuclease at 37 °C for 1 hour. The digested DNA was incubated at 37 °C for 2 hours with Klenow enzyme and Primer A to generate

complementary DNA with a discrete 5' terminal. Next primer B and Qiagen Hotstart Taq polymerase was used to amplify the generated cDNA and label it with amino-allyl dUTP for 40 cycles using the following profile: 30 seconds at 94°C, 30 seconds at 40°C, 30 seconds at 50°C, 60 seconds at 72°C for 40 cycles.

Labeled DNA and RNA were coupled separately with Cyanine dye 3 and Cyanine dye 5. This reaction was carried out in the absence of light and using 1 molar sodium bicarbonate (pH 9.5) as a coupling buffer with a 1-hour incubation. Following coupling, uncoupled dye was removed using the QIAgen PCR purification kit and the labeled virus DNA and RNA were dessicated in a DNA 120 SpeedVac (Thermo Fisher Scientific, MA, USA) for 1.5 hours. The sample preparation, labeling and hybridization procedure is outlined in Figure 5.1.

6.3.5 Hybridization of Samples

Microarray hybridization was performed as described previously (Wick et al., 2006). The microarrays were hybridized and washed in a M-2 microfluidic station (Invitrogen, Carlsbad, CA, formerly Xeotron Corporation, Houston, TX) using a flow rate of 400 microliters / minute. The hybridization buffer was 6x· SSPE, 25% formamide, 0.4% Triton X-100. Chips were pre-hybridized with 6x· SSPE, 0.2% Triton X-100 and then with hybridization buffer for 2 min each.

Labeled targets were resuspended in 50 microliters hybridization buffer, denatured at 95°C for 3 minutes, cooled on ice for 1 minute, filtered through a 0.22 micrometer Costar spin filter and then hybridized to the chip for 14–15 hours at 20°C.

The chip was scanned with a GenePix 4000B laser scanner (Axon Instruments, Union City, CA). All solutions were filtered through a 0.22 micrometer filter to prevent clogging of the microfluidic channels. The high stringency wash buffer was degassed under vacuum. All arrays were imaged with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) and GenePix Pro software.

6.3.6 Data Analysis Using DetectiV

The DetectiV software package was used to visualize, normalize and test the significance of the microarray hybridization data (Watson et al., 2007). DetectiV was used to generate bar plots of the viral microarray signals following normalization against the median signal values for each sample. DetectiV was also used to carry out statistical t-test comparisons of the hybridization signal values between different virus groups in order to determine which groups of virus target had statistically significant probe signals.

6.4 Results

6.4.1 Viruses Present in January 2008 Control Sample

Using DetectiV and applying the detection criteria of p value less than or equal to 0.01 and log average signal greater than or equal to 0.5, five virus groups were identified

in the January 2008 control sample (sample with no poliovirus). The five groups of viruses were Human adenovirus type 41, Human astrovirus, Human adenovirus group E, Human enterovirus group D and Human enterovirus group A. Table 6.1 lists the p values and log mean signal values for the ten most likely targets for the January 2008 sample.

6.4.2 Sensitivity Testing using Spiked Poliovirus LS-C-1

The sensitivity of the microarray was tested using a range of concentrations of poliovirus LS-C-1. A ten-fold serial dilution of poliovirus stock to give final concentrations of poliovirus of between 1.2 and 12000 plaque forming units resulted in $\log_2$ average signal readings for poliovirus of between 0.258 and 1.237 when DetectiV was used to analyze the hybridization results. This translated into a detection sensitivity for the array of approximately 59 poliovirus plaque forming units in order to achieve a $\log_2$ mean signal intensity of 0.5 in this sample. Figure 6.1 shows the plot of $\log_2$ average signal against poliovirus plaque forming units.

Table 6.1 p-values and log_2 average signal for the ten most likely viruses present in the January 2008 sewage sample

Virus	p value	Log_2 average signal
Human adenovirus type 41	0.0003939283	0.7321386
Human Astrovirus	0.0008543337	0.9832780
Human Adenovirus E	0.0038398798	0.3784977
Human Enterovirus D	0.0056843549	0.5509884
Human Enterovirus A	0.0062747645	0.7452414
JC Polyomavirus	0.0076384783	0.2057239
BK Polyomavirus	0.0141987829	0.2330732
Human Enterovirus E	0.0160536991	0.3271534
Sapovirus	0.0162622777	0.2643265
Human Adenovirus D	0.0174830642	0.3644236

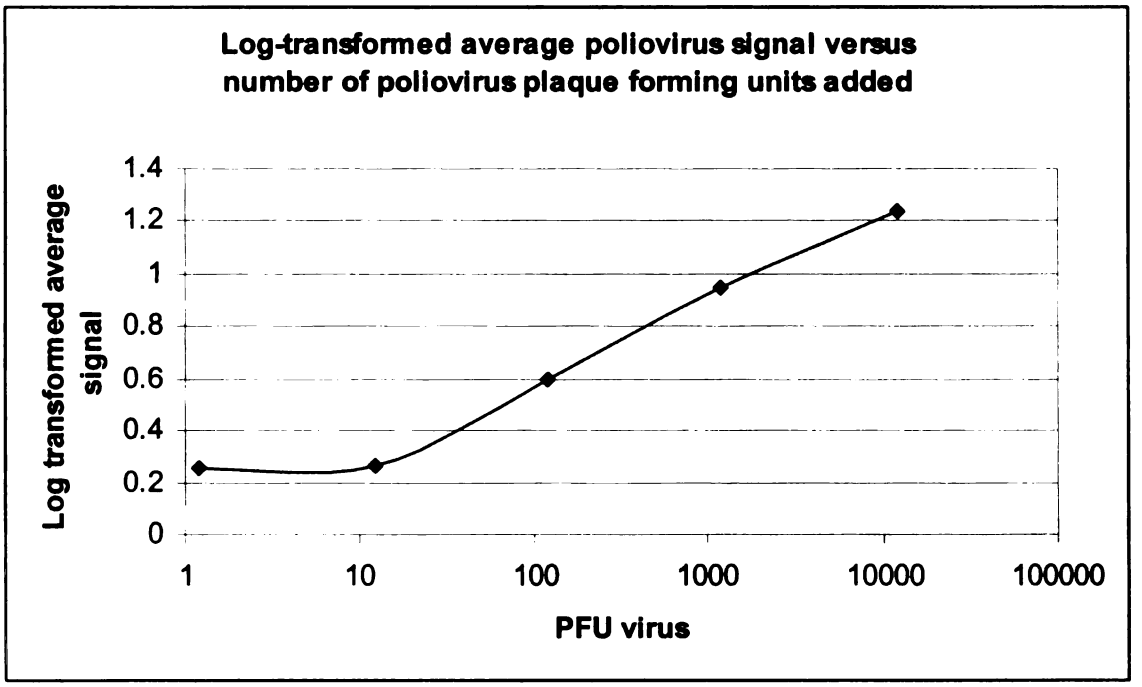


Figure 6.1. log_2-transformed average poliovirus signal versus number of poliovirus plaque-forming units (PFU). PFU virus (x axis) is provided in logarithmic scale.

6.5 Discussion

6.5.1 Viruses present in January 2008 sewage

From the results, the January 2008 sewage sample contained 5 types of viruses – Human adenovirus type 41, Human astrovirus, Human adenovirus group E, Human enterovirus group D and Human enterovirus group A. This was similar, though no identical, to the numbers and types of viruses obtained in the previous year (January 2007) – Human astrovirus, Human enterovirus group A, Human enterovirus group D, Human enterovirus group E, norovirus, sapovirus, Human adenovirus group B and Human adenovirus type 41 signals were observed in that sample. Out of the 5 groups of viruses present in the January 2008 sample, 4 were also present the previous year. Human adenovirus group E was present in the January 2008 sample but absent in the January 2007 sample. Human enterovirus group E, Human adenovirus group B, noroviruses and sapoviruses were present in the January 2007 sample but absent in the January 2008 sample.

Three groups of viruses, Human enterovirus group A, Human astrovirus and Human adenovirus type 41, were present in 4 samples collected during winter months (December to February). These three groups of viruses might represent viruses that are either constantly being circulating within the community or else representing an endemic presence in the community. Table 6.2 illustrates the distribution of virus groups among the 4 samples collected during winter months. Two groups of viruses were present in two of the four months, Human enterovirus group E was present in the December 2006 and January 2007 sample. Human enterovirus group D was present in the January 2007 and

January 2008 sample. These might reflect groups of viruses that are less widespread among the community during winter months. Four groups of viruses were present in only one of the 4 samples, norovirus, sapovirus, Human adenovirus group B and human enterovirus group B. These might represent viruses that are only transiently present in the community.

6.5.2 Spiking sensitivity

Based on the results from spiking poliovirus in raw sewage, the microarray showed a sensitivity of approximately 59 plaque forming units in order to achieve a $\log_2$ mean signal intensity of 0.5, which was the detection criteria used in this study. Compared to reverse transcription polymerase chain reaction and other reported methods, the detection limits reported in this study were low. Polymerase chain reaction detection of viruses in environmental water was widely reported to be at least a hundred-fold higher. The sensitivity of the array could potentially be improved (i.e < 59 plaque forming units) if the sample contained fewer types of target viruses as there would be less competition for labeling and hence more signal from the viruses that are present. Alternatively, if specific primers were used for labeling target individual target viruses. Sensitivity tests would have to be carried out for different water matrices to determine the effect that target concentration vis-à-vis background concentration of viruses would affect signals on the array.

6.5.3 Conclusions

Based on the sensitivity test using poliovirus spiked in sewage, the virus microarray is able to detect the presence of viruses at concentrations commonly present in sewage.The intensity of the signal however is potentially affected by the presence of inhibitors for the labeling of viral nucleic acid, the coupling of the florescent dye to the label nucleic acid, and the proportional concentration of the target virus in relation to the background nucleic acid levels which serve as competition for labeling. Inhibition issues were partly avoided by performing a cell culture incubation step prior to virus labeling and hybridization. This would help to biologically amplify the virus and dilute any inhibitors that might be present in a sample.

In addition, it was observed that three of the winter month samples analysed during the the year-long survey of raw sewage and one other sample collected in January 2008 used for the sensitivity test for poliovirus spiked in sewage showed the presence of three groups of viruses that were present in all four samples. This might indicate that these viruses were constantly circulating among the community during the winter months and also potentially endemic to the community since they were present in a sample taken a year later. This illustrates one potential use of the virus microarray as a means of monitoring a community's wastewater virus signature in order to gain insights into the range of viruses that are continuously circulating within a community and groups of viruses that are found more transiently within human wastewater.

Table 6.2 Distribution of virus groups among winter month samples

Month	Virus group present in all 4 winter months	Virus group present in 2 out of 4 winter months	Virus group present in 1 out of 4 winter months
December 2006	Adenovirus type 41 Human astrovirus Human enterovirus group A	Human enterovirus group E	
January 2007		Human enterovirus group E Human enterovirus group D	norovirus sapovirus Human adenovirus group B
February 2007			Human enterovirus group B
January 2008		Human enterovirus group D	

CHAPTER 7 SUMMARY AND CONCLUSION

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, 1989). Yet it had long been understood that enteric viruses such as poliovirus were shed in the 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 United

States 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).

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, poor infrastructure, 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 which 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 sewerage 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* have 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 Immuno Deficiency 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 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) which are used for the detection of viruses. Even though many viruses are culturable in several cell lines and are thus detectable by their development of CPE cell culture, there are several viruses, like enteric waterborne adenoviruses types 40 and 41, which 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 in some cases the specificity and can be labor-intensive and time-consuming. However, detection of infectious viruses and ability to process relatively large volumes of concentrates (up to 30 ml) means greater sensitivity.

The limitations of the cell culture method were highlighted in Chapter 4. We found that PCR detected more positive virus samples than conventional cell culture (30 positives and 26 positives respectively out of 58 samples tested). We attributed this disparity to the presence of non-CPE forming enteric viruses in the sample. In addition, in our study we observed that while higher number of adenovirus and rotavirus-positive samples were detected at the Silver Beach location compared to the Washington Park location, the enteroviruses and enterococcus surface protein (ESP) PCR results would have led to the opposite conclusion. In our study we proposed that the contradictory results could be explained by the different survival rates of the different indicators, underscoring the need to screen any environmental sample against multiple target indicators before reasonable conclusions can be made. Lastly we observed that while a positive virus PCR result could be correlated with a positive cell culture result ($\chi^2 = 4.66$; 1 degree of freedom), not all the cell culture positive samples could be identified through PCR. We believe that while the inability to assign identities to these samples using the PCR primer sets we had available could be attributed to some extent by the presence of inhibitors during the polymerization chain reaction in the sample (as documented through realtime PCR analysis of the same set of samples by Xagoraraki et al. (2007)) it is also

likely that the inability to achieve a PCR identification of these samples could also be due to the presence of cultivatable viruses that we did not have primers for.

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 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 adopt a more proactive preventative role instead of merely recording and reporting disease data. In order to do so, there needs to be tools which 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.

In the microarray experiments (Chapter 5), it was demonstrated that oligonucleotide microarrays provide an approach for screening hundreds of pathogens in environmental samples. While microarrays have previously been used to perform environmental analyses, namely describing microbial communities and elucidating levels of gene expression among microbial consortia, use of this platform for the detection of viruses in polluted waters, which cause human disease represents a novel use of this technology. This is especially important because unlike many of the other fecal indicators, no suitable indicators of viral fecal pollution have been forthcoming. In this

research, a community's virus infection profile was characterized through a series of samples taken over a period of 13 months. RNA viruses were more prevalent than DNA viruses, and there was some degree of seasonality. The next steps are to begin examining disease in the community in relationship to this biomonitoring of the infection in the population.

In addition, we were able to validate two pieces of software, one used to design the probes for the microarray and the second used to visualize and validate the hybridization signals generated by the arrays. The high degree of specificity demonstrated by the virus microarray chip from the very onset in hybridization experiments with laboratory and commercially purchased strains of viruses demonstrates that the OligoArray 2.1 software is extremely proficient at selecting probes with a high degree of specificity under the conditions set. The other piece of software, DetectiV, has previously only been applied to analyze hybridization signals generated during the analysis of clinical specimens for the presence of viruses. Nevertheless the broad applicability of the program has been demonstrated by our use of this software, with only a few modifications to the acceptance criteria, to analyze signals generated from environmental samples which are inherently more complex compared to patient clinical samples.

Chapter 6 describes the study in which poliovirus was spiked into the cell culture supernatant of African Green Monkey kidney cells that had been exposed to raw sewage concentrates. This study was used to determine the level of sensitivity needed to produce

a positive signal on the microarray. In this study, the sensitivity of the virus microarrayto poliovirus spiked into sewage was found to be approximately 59 plaque forming units in order to produce a $\log_2$ mean signal of 0.5. This level of sensitivity is reflective of the concentration of viruses commonly found in less than 100 milliliters of raw sewage. Also Chapter 6 reports the observation that three groups of viruses, Human adenovirus type 41, human astroviruses and Human enterovirus group A, were present in all 4 samples collected between December 2006 and February 2007 as well as January 2008. This suggests that these three groups of viruses are either endemic, since they occurred in samples that were taken as much as a year apart or that they are widely and continually being spread among the community.

The objective of this dissertation is to compare the current methods used to test for viruses in the environment, namely cell culture, polymerase chain reaction (PCR) and integrated cell culture-PCR against the results obtained using a microarray to detect viruses through labeling and hybridization post cell culture. Our analysis of the cultivatable and PCR-identifiable viruses collected from two recreational beaches along the Great Lakes indicates that this is likely to be so. We have shown that conventional cell culture is limited in its ability to detect a wide range of viruses due to the host specificity of most animal viruses. We have also documented that conventional molecular detection methods like the polymerase chain reaction suffer from inhibition problems and are extremely specific in their target range, making high throughput screening of samples for any one or a combination of the more than a hundred different varieties of human and animal viruses impossible. Modifications to the conventional cell culture method have

helped to overcome some of these issues. One such modification is to pair cell culture with the polymerase chain reaction (PCR) in order to over come inhibition issues as well as to increase the speed and sensitivity of the detection of viruses in environmental samples. This thesis describes the next evolution for virus detection in which a microarray hybridization step is incorporated post cell culture in order to increase the throughput for virus testing, overcoming the current limitation of the cell culture-PCR method. Using the virus microarray, we have demonstrated that oligonucleotide arrays are able to hybridize with complex, mixed environmental samples and may be used as a multiple pathogen detection/screening tool.

Our analysis of human wastewater samples with the microarray indicate that a wide range of DNA and RNA viruses are present in human feces and could potentially survive to reach the environment if improperly treated. 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 concentration 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 are all issues that deserve future attention and elucidation. 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.

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