OCCURENCE OF ENTERIC VIRUSES ON COMBINED SEWER OVERFLOWS

by

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DEDICATION

To my wife Damaris

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ABSTRACT

The goals of this dissertation were to determine the viral contribution of combined sewer overflows (CSO) to receiving waters during wet weather conditions, and to compare the use of the primary liver carcinoma (PLC/PRF/5) cells with the buffalo green monkey (BGM) cells for total culturable virus assay (TCV). To assess the contribution of CSO on the viral quality of the receiving water, samples of discharges and effluent receiving waters of three sewage reclamation plants located in Illinois were collected from June to October during two consecutive years. Samples were tested for TCV, adenovirus and norovirus. Viral concentration in the receiving water increases approximately ten times the concentration during CSO events in comparison with dry weather. An assessment comparing TCV by PLC/PRF/5 and BGM cells was also conducted using sewage samples collected before and after disinfection. PLC/PRF/5 cells detected between 10 to 50 times more viruses (?) than the BGM cells. Adenoviruses were detected in the PLC/PRF/5 cells, but not in the BGM cells. In conclusion, CSO events resulted in a significant increase in the concentration of viruses in the receiving waters and PLC/PRF/5 cells are more sensitive for enteric virus detection than the BGM cell line.

CHAPTER 1. INTRODUCTION

Enteric viruses have been detected in surface, ground and drinking water (1, 2, 5-7, 14, 17, 22, 22, 24, 29). Bacterial indicators of water quality are commonly used for monitoring the efficacy of water treatment facilities and the quality of water. However, they are not correlated with the occurrence of enteric viruses (4, 18). Therefore, it is useful to assess enteric virus occurrence in water.

Sewer systems in older cities in the United States are usually combined with storm drain systems. During wet weather, the amount of combined sewage with storm water runoff surpasses the treatment capacity of wastewater treatment plants and forces the release of the overflow of untreated sewage into receiving waters resulting on the release of human pathogens (3, 12). Alternatives to reduce sewer overflows include the construction of sewage storage, the use of artificial wet lands, and the use of high rate treatment systems (25, 27, 30, 31). The evaluation of technology needed for treatment of wet weather effluent will be affected by the characteristics of the sewage and by the load of pathogens in the sewage. Therefore, the characterization of viral pathogens in CSOs is necessary to assess the risk if these waters are used for recreational proposes and the safety of shellfish growing areas.

Cell culture is the standard for the detection of enteric viruses in water (26). Limitations and advantages of the use of cell culture are listed in Appendix A. Cell lines vary in their susceptibility to infection by different viruses (10). Buffalo green monkey (BGM) cells are a very sensitive cell line for the detection of poliovirus and Coxsackie virus B (9). Primary liver carcinoma (PLC/PRF/5) cells are very sensitive for the

detection of adenovirus (15). Human colon carcinoma (Caco-2) cells are very sensitive for the detection of astrovirus and many other viruses (21). Many cell lines will grow several types, but usually not all the enteric viruses. For example, the Caco-2 cell line can be used to isolated astrovirus, adenovirus, rotavirus, and enteroviruses (21); on the PLC/PRF/5 cell line, adenovirus, enteroviruses, and reovirus (16); and on the BGM cell line, enteroviruses, simian rotavirus, and adenovirus (9, 20). Therefore, the use of only one cell line can underestimate the total number of infectious viruses present in the water sample (8, 23).

One limitation in the use of cell cultures is that some viruses such as hepatitis A viruses and norovirus are difficult to propagate (13). The Polymerase Chain Reaction (PCR) is the most commonly used method for the detection of non-culturable viruses. In addition, PCR has been widely used for the detection of viruses in water because the results can be obtained in a short period of time and the cost per sample is reduced in comparison to the cost of analysis by use of cell culture. Appendix 1 lists the limitations and advantages of the use of PCR for the detection of viruses from the environment. Also, Appendix 1 reviews attempts with PCR to assess viral infectivity.

This dissertation is composed of three manuscripts. The first manuscript is a literature review of the application of PCR for assessing viral infectivity. The second is in the use of PLC/PRF/5 cell line in the evaluation of alternative treatment of combined sewage overflows.

CHAPTER 2. PRESENT STUDY

Appendix A reviews attempts to use the polymerase chain reaction to assess viral infectivity. To date, no literature review has been published on this topic. Although cell culture is the standard for the detection of infectious viruses from the environment (10), some important waterborne pathogens such as norovirus cannot be detected using cell culture (11), leaving the use of PCR as the best alternative for their detection. The assessment of infectivity for this non-culturable virus is very important because it is the major cause of viral gastroenteritis in the United States. In addition, it is important to understand the detection of viral genome in terms of risk of infection.

The study presented in Appendix B demonstrated that the primary liver carcinoma cells (PLC/PRF/5) were more suitable for the detection of enteric viruses from sewage than buffalo green monkey kidney cells (BGM). BGM is the standard cell line for the detection of enteric viruses from water (26). The use of multiple cell lines has demonstrated the detection of viruses that do not grow well in the BGM cell line and the exclusive use of BGM cells may greatly underestimate the numbers of enteric viruses in a sample (16, 19, 23, 28). The study presented in Appendix B shows that the PLC/PRF/5 cells are between 10 to 50 times more sensitive than the BGM cells for the detection of infectious viruses in sewage. Viruses such as adenovirus are detected in the PLC/PRF/5 cell line and not detected in the BGM cell line.

Appendix C compares the viral concentration of undisinfected sewage in receiving waters during wet weather and dry periods. The concentrations of the total cultivable viruses (TCV), infectious human adenovirus and human norovirus, were

monitored in the receiving waters for a period of 18 months. In conclusion, we demonstrated that when combined sewer overflows occur during wet-weather events, the viral concentration increases on average up to ten times the concentrations observed during dry weather. This is true for TCV, infectious human adenovirus, and human norovirus.

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APPENDIX A

THE APPLICATION OF POLYMERASE CHAIN REACTION-BASED METHODS TO ASSESS THE INFECTIVITY OF ENTERIC VIRUSES IN ENVIRONMENTAL SAMPLES

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INTRODUCTION

The advent of the polymerase chain reaction (PCR) has greatly enhanced our ability to detect human enteric viral pathogens in the environment including water, municipal wastes, sewage, food, air and fomites (2, 3, 44, 50, 60). This is especially true for viruses, which do not grow in cell culture. Despite great sensitivity, PCR methods do have some serious limitations for environmental viral analysis (49). Among these are: small sample volumes, the presence of PCR inhibitory substances and inability to differentiate between viable and non viable viruses. The ability of PCR to assess viability would greatly enhance its application in environmental virology. This review focuses on approaches to overcome this limitation.

DETERMINATION OF VIRAL INFECTIVITY

Viral infectivity can be described as the capacity of the virus to enter the host cell, and use cell resources to ultimately produce infectious viral particles (virion) (9). The virion of most enteric viruses is composed of two major components, the capsid and the genome (63). The protein capsid is involved in the interaction of the virus with the host cell surface and has antigens specific to cell receptors used to enter the cell. The capsid also has the function of protecting the viral genome from degradation by nucleases and environmental stresses such as humidity, pH, UV radiation, and temperature. Thus, an undamaged viral capsule is critical for the initiation of a successful infection

In addition to the viral capsule, the replication and translation of the viral genome to viral proteins and enzymes are also important during a successful infection (63). The

properties of the genome vary among the different groups of enteric viruses including positive stranded RNA viruses, double stranded RNA viruses, and double stranded DNA viruses. Therefore, each viral group has its own mechanism for translation and replication of the genetic information. Only positive stranded viruses can initiate an infection by means of intact naked viral RNA without the viral capsid. However, this is very difficult and inefficient; in the case of poliovirus only one naked positive strand of RNA in ten thousand can start an infection (59).

Standard methods for the detection of infectious viruses in water require the use of susceptible cell lines within which the viruses can propagate and produce cytopathic effects (CPE) and be visualized under the light microscope (16). It is important to emphasize that even using cell culture, the detection of infectious viruses from the environment is difficult. Each virus has different capabilities to propagate in each cell line. For example, not all enteroviruses can propagate effectively in any one cell line (14), which means that the detection of all the enteroviruses in water may require the use of multiple cell lines (52). In addition, detection of infectious viruses in a sample will greatly depend the assay conditions i.e. duration of exposure to host cells, volume of inoculums, age of the cells, etc.

The advantages and disadvantages of cell culture for viral detection are listed in Table 1. One important limitation is that some viruses, such as norovirus and hepatitis virus are difficult to grow in the cell lines currently available. Detection of norovirus in particular relies largely upon direct PCR of environmental samples, which does not provide information on infectivity (19, 32). Addressing the infectivity of slow growing

or non-culturable viral pathogens is very important in understanding their persistence in the environment, the efficacy of disinfection, and ultimately the estimation of the risk of transmission to susceptible human populations.

DETECTION OF VIRUSES BY DIRECT RT-PCR/PCR

PCR based methods have been successfully used to monitor water and food products for viral contamination (3, 6, 7, 13, 18, 20, 34, 62). During PCR assay, a fragment of the viral genome is amplified using specific primers. For RNA viruses, translation of viral RNA to DNA via reverse transcriptase is necessary prior to PCR assay. This is not necessary for viruses like adenoviruses, which contain only DNA sequences. A specific set of primers is designed for the detection of each particular virus. However, regions or genes in the viral genome can be conserved among families of viruses, which allows for the use of a primer set that can detect multiple members of a particular viral family. For example, a region of the adenovirus genome that codes for the production of the capsid hexon protein can be used for the detection of human adenovirus such as types 2, 40 and 41 (5). Mutiplex PCR, which utilizes multiple primer sets within a single PCR reaction, to detect different groups of viruses. However, this multiple viral detection can be difficult to optimize because of the different annealing temperature requirements of different primer sets, and because of the difference in properties of the viral nucleic acids found between viral groups (20, 21). In some cases, further confirmation steps such as oligonucleotide hybridization are needed in order to confirm the specificity of the detection (21).

The advantages and disadvantages of PCR for the detection of viruses in the environment are listed in Table 1. In the case of viruses that grow poorly in cell culture the detection by PCR or by the combination of cell culture and PCR drastically reduces the time needed for detection (47)

Because viruses are normally present in very low concentrations in ground or surface waters, large volumes of water must be tested. In the VIRADEL (virus absorption and elution) method, large volumes of water (100 to 1000L) are passed through a charged filter, and the virus absorbs to the filter matrix due to its electrostatic charge. This is followed by virus elution from the filter and re-concentration to a final volume of 25 to 35 mL (45). In neutral pH waters, the virus is negatively charged, and can be absorbed using an electropositive charged filter such as 1-MDS. The viral sample is usually eluted from the filter matrix using a slightly alkaline solution of beef extract. When beef extract is used for viral elution, the sample can be re-concentrated by protein flocculation at low pH. During flocculation, the pH of the eluted sample is lowered to 3.5, the beef extract produces a floc, which is pelleted by centrifugation and the virus is re-suspended in 25 to 35 mL of buffer at pH 7.5.

Another disadvantage of direct PCR is the limited sample volume that can be assayed (49). Normally the volume of the PCR reaction mixture is between 50 to 100 μL, which limits the sample sizes between 10 to 30 μL. Using large volume PCR, the sample volume can be increased to 100 μL (3). In contrast, with cell culture it is feasible to assay 30-50 mL. The equivalent volume of the original sample using PCR often represents less than one to 10 L from the original sample processed. For this difference

in sampling volume, the actual sensitivity of PCR can be less than the sensitivity of detecting viruses directly in cell culture (49). The VIRADEL method typically yields concentrates of 25-35 mL (41), which can be concentrated down to 100 μL by using microconcentrators (64). While this increases the sensitivity of PCR, inhibitory substances are also concentrated which can negate the additional volume reduction.

Comparison of the sensitivity of direct PCR and cell culture assay is difficult because of the ratio of infectious viruses to viral particle is variable. In the case of rotavirus in the MA104 cell line, the ratio can be cell cultivable virus to 40,000 viral particles (67) and in the case of adenovirus using PLC/PRF/5 cells the ratio can be around one in 1,000 (26). This ratio infectious/viral particle is largely dependent on the assay method and how long the virus has been passed in the particular cell line. Thus, viruses from direct clinical or environmental samples have a much higher ratio than those viruses that have been adapted to cell culture (66)

In the case of quantitative-PCR, an internal control can be used to measure the amount of inhibitors in the sample (28), allowing for the identification of false negative samples. The treatment of the sample with resins or commercially available kits can be used to aid in the removal of inhibitors such as humic acids (1). However, some virus may be loss during any purification process and simple dilution of the sample may yield the same result.

DETERMINATION OF VIRAL INFECTIVITY USING PCR

The viral genome is protected by the viral capsid. Damage to the viral capsid may result in the lost of its capacity to protect the viral genome and in the lost of virus ability

to replicate in the host. The detection of an intact genome can be an indication that the virus capsid is still in good condition, protecting the genome from degradation. Determining the relationship between damage to the viral capsid and degradation of the viral genome will provide information that could be use to correlate the detection of viral genome with the infectivity of the virus. PCR or RT-PCR then can be used to detect damage in the target region of the viral genome (66), e.g. damage of the target region of the nucleic acid may result in failure of the PCR reaction (or RT-PCR). Methods, which use this approach, are summarized in Table 2.

Treatment with Proteases and Nucleases before PCR

As discussed previously, one function of the viral capsid is to protect the nucleic acids from degradation by nucleases found in the environment. However, a damaged viral capsid is more susceptible to proteases than an intact capsid. The degradation of the viral capsid by protease will eventually expose the viral nucleic acid to nucleases. Protease and RNAse pre-treatment can be used to differentiate between an intact virus and a virus that has been inactivated by disinfection (41). This is because the protease pretreatment will degrade the capsid that has been damaged during disinfection and the nuclease pretreatment will then degrade the unprotected nucleic acid yielding a negative PCR result. This approach has been successfully used to determine the effectiveness of ultraviolet light disinfection, chlorine disinfection, and thermal treatment at 72°C in the inactivation of hepatitis A virus, poliovirus 1, and feline calicivirus (36). The use of this enzymatic pretreatment has failed to discriminate between infectious viruses and viruses inactivated at 37°C (41).

Immuno-separation, cell culture attachment and PCR

The viral capsid has antigens that are used by the immunologic system to produce specific antibodies against viruses during infection (63). The antigenic properties of the viruses can be used for the production of specific immunoglobulims. Immunoglobulins can recognize the viral antigen and attach to it, forming and antigen-antibody complex. Immunoglobulins are commonly used for the detection of viruses by the Enzyme-Linked ImmunoSorbent Assay (ELISA) and other immunological techniques in clinical laboratories. Immuno-magnetic separation (IMS) has been commonly used for the concentration of enteric protozoan pathogens and viruses such as noroviruses and enteroviruses from water samples (17, 25, 52). In this technique, the antibodies are attached to a surface or to a paramagnetic bead. The target pathogen attaches to the antibody and is then removed from solution. The target pathogen can then be released from the antibody and assayed by PCR. The main advantage of this technique is that the concentration step is specific and inhibitors are not co-concentrated. The use of IMS in samples with high concentrations of humic acids has demonstrated this capacity (40). One limitation is that monoclonal antibodies can be too specific and only one serotype of virus can be isolated making it necessary to create a broad range of antibodies for the detection of the various groups of enteric viruses (25).

Although the combination of immuno-capture with PCR seems to have the potential to detect damages in the viral capsid, it has been reported that this technique does not have the sensitivity to discriminate between infectious viruses and viruses

inactivated with UV, chorine and heat treatment (42). In addition, it has been reported that UV inactivation did not change antigenic properties of hepatitis A virus (66).

Determination of viral attachment to the host cell and PCR

Nuanualsuwan and Cliver (42) studied interference with virus attachment to cell monolayers as a way of assessing viral inactivation by UV, hypochlorite and heat. They demonstrated that inactivated viruses do not attach to cell monolayers and can be easily removed by rinsing the monolayer after incubation with the virus. PCR analysis of the cells demonstrates the presence of infectious virus. This approach was successfully used with poliovirus type 1, hepatitis A and feline calici virus using the cell lines in which they were propagated (42). It would be interesting to determine if the same results can be obtained in cell lines in which the viruses cannot propagate effectively. For example, an infectivity assay has been recently described for the propagation of noroviruses, but this assay requires a cell line that is not widely available and special cell culture techniques making it difficult to use on regular basis (61). The use of cell attachment and PCR may be a practical alternative for the analysis of disinfectant effectiveness in the inactivation of norovirus because it may not require the cell differentiation processes necessary for propagation.

The Use of Long Target Region for PCR (LTR PCR)

LTR PCR is an approach than can be used to detect damage in the viral genome and is based on the theory that the larger the fragment of the viral genome analyzed, the greater the probability of detecting damages in the genome using PCR. In this approach

damages to the nucleic acid which inhibit the PCR reaction are detected. During the amplification step, damage in the amplified region yields a negative PCR result. Simonet and Ganzer (56) compared the size of the target sequence amplified and capacity to detect changes in the RNA of poliovirus after UV irradiation. They found that the larger the genome region analyzed, the more damage to the nucleic acid that was detected. For example, only a 0.9 $\log_{10}$ reduction in amplifiable genome was obtained after a radiation dose of 150 mJ of UV when using a primer set that amplifying a region of 149 bases using quantitative PCR (qPCR). On the other hand, a 1.0 $\log_{10}$ reduction was observed with a lower radiation dose (20 mJ) when using a target region of 1,869 bases. However, a 3 $\log_{10}$ reduction in infectious viruses was observed when the a radiation dose of 20 mJ was used.

One disadvantage with this approach using qPCR may be the difference in amplification efficiency between the amplification of short and long fragments. Pre-amplification with a long fragment and amplification with an internal short fragment should reduce this difference (4).

Targeting the 5' Non-Translated Region (NTR) with PCR

The Picornaviridae family are positive strand viruses and have a cap independent mode of translation, the 5' non translated region (NTR) is very important in the translation of the RNA to proteins. It contains sequences and secondary structures such as the internal ribosomal entry site (IRES) necessary for translation (63). The 5' NTR has been reported as the most sensitive region on the genome of hepatitis A virus (HAV) to degradation upon exposure to chlorine and chlorine dioxide (8, 36). The lack of

amplification of the 5' NTR accompanied the loss of viral infectivity in cell culture. The first 600 bases of HAV genome were more sensitive to chlorine degradation than the rest of the genome (36). Simonet and Gantzer (56) analyzed the kinetic of poliovirus genome degradation using a qPCR approach during exposure to chlorine dioxide reach similar conclusion. They also found the use of LTR-PCR increased the sensitivity of the assay to detect degradation of the genome. For example, one log reduction was observed for an amplified fragment of 145 bases of the 5' NTR after 15 minutes of exposure to 5 mg/L of chlorine dioxide. Under the same conditions and in the same region in the 5' NTR, a 3.0 $\log_{10}$ reduction was observed when the amplified fragment was 6980 bases long. However, the reduction of infectivity was 4.5 $\log_{10}$ after 3 min of exposure to 5 mg/L of chlorine dioxide, which indicated that even with the use of a longer region as a target for the PCR reaction, reduction of poliovirus was underestimated. In addition, the first 400 bases of the poliovirus genome were not included as the target region (55), which could have increased the sensitivity of the assay in determining genome degradation.

DETECTION OF VIRUSES USING A COMBINATION OF CELL CULTURE AND PCR (ICC/PCR)

Cell culture combined with PCR (ICC/PCR) is an approach that has been used to overcome most of the disadvantages associated with conventional cell culture, and those associated with direct PCR assays (46). In this method, detection relies on biological amplification of viral nucleic acid, followed by enzymatic amplification via PCR (48). Hence, virus is allowed to replicate in cell culture for short periods of time followed by PCR analysis, which dramatically reduces the time necessary for viral detection (46).

The advantages, disadvantages and approaches to ICC/PCR are summarized in Table 3. ICC/PCR also has the advantage of detecting viable viruses that do not produce CPE. The sensitivity obtained in ICC/PCR is comparable with the sensitivity obtained in cell culture after a second passage of the sample in cell culture (10), reducing the time needed to obtain a sensitive detection of infectious viruses. In addition, fewer problems are encountered with inhibitory compounds contained in environmental concentrates (12) .

The use of ICC/PCR has been described for the detection of enteroviruses (48), hepatitis A virus (31, 47), enteric adenovirus (34) and astrovirus (29). The integrated use of cell culture with PCR has demonstrated a wide distribution of infectious viruses in water sources since it allows for the detection of non-CPE producing enteric viruses (35, 47). Lee et al. (35) demonstrated that enteroviruses and adenovirus could be detected simultaneously in the same cell line with this approach.

Detection of Viral Nucleic Acid Intermediates during Infection

During infection, the viral genome is transcribed to mRNA or other intermediary in the host cell, which is eventually used for synthesis of viral proteins or replication of the genome (63). These steps are essential for viral replication. The detection of these intermediaries in the host cell during infection is a clear indication that the virus is replicating in the host cell, and is infectious.

In a positive strand RNA virus, the primer used is complementary to the sequence of the negative strand, the negative strand is transcribed to cDNA and then amplified by PCR (31). During infection, the HAV positive strand is transcribed to a negative strand in the host cell. This negative strand is used to produce more positive strands that

eventually are packaged in the viral capsule or used as a template to produce more viral mRNA. Negative strand RNA is a complementary copy, and in order to be used as mRNA, needs to be transcribed to a positive strand. The HAV virion does not contain negative strand RNA and the detection of viral negative strand RNA is a clear indication of infection.

The specific detection of the negative strand uses a primer that targets a complementary sequence of the positive strand. This primer allows for the specific reverse transcription of the negative strand to cDNA, which is used as a target for PCR. Strand specific RT-PCR has been used in clinical studies for the detection of infectious hepatitis C from biopsy samples (51) and in demonstration of the replication of enterovirus in valvular tissues from patients with chronic rheumatic heart diseases. This technique has been described for the detection of hepatitis A virus using ICC/RT-PCR (31).

Detection of HAV using the ICC/strand specific RT-PCR, depends upon the negative strand being detected in the cell extract after a successful infection. The sensitivity of HAV detection using ICC/strand specific RT-PCR is one $TCID_{50}$/mL per cell culture flask within 4 days of incubation (31). This study demonstrated the possibility of a false positive result when the concentration of inactivated viruses virus in the sample exceeds 10^3 $TCID_{50}$/mL. The same principle is used in the detection of adenovirus via mRNA RT-PCR (33). During infection, the adenovirus DNA needs to be transcribed to mRNA and the mRNA translated to functional proteins (i.e. DNA polymerase), as well as, non-functional (capsid proteins) proteins. Since double-stranded

DNA is a very stable molecule, it is possible to detect DNA from non-infectious virus without the propagation of the virus in the host cell if the sample analyzed has a high concentration of adenovirus. In the detection of adenovirus using a combination of cell culture and RT-PCR, the detection of mRNA of adenovirus is a clear indication of viral infection because the viral mRNA is only detected in the host cell during the infection. Two sets of primers were used by Ko et al. (33) one for the early gene EA1, and another set for a late hexon gene (capsid protein) for the detection of adenovirus 2 and 41. They found that the sensitivity of the mRNA detection varies between the serotype and genes. The sensitivity of the assay after 7 days of infection was 0.2 infectious units (IU) for adenovirus 2 using the mRNA of E1A gene and 0.1 IU for adenovirus 41 using the mRNA of the hexon gene. The authors also compared the impact of chlorine and UV light disinfection on detection by cell culture and PCR. The copy numbers of mRNA for the hexon gene in the cell can reached 10^5 copies after 36 hours of infection. This high ratio of viral mRNA to viral DNA results in an increase in the sensitivity of the assay (33).

THE STABILITY OF VIRAL GENOME AND ITS RELATIONSHIP WITH VIRAL INFECTIVITY IN WATER

Direct PCR analysis of water samples has become common during the last decade. Horamoto et al. (30) found that up to 7 % of samples from tap water in Japan contained the norovirus genome. In addition, the norovirus genome has been detected in surface (38) and ground waters (11). Rotavirus and adenovirus genomes have also been

detected in drinking water (60). However, the detection of viral genomes may not be an indication of the risk of exposure to an infectious virus. Therefore, understanding the relationship between a viral genome and viral infectivity will be very valuable for the interpretation of PCR results. Table 4 summarizes studies which compare the detection of infectious viruses with the detection of viral genome in various types of water.

However, the interpretation of PCR results with results obtained by cell culture in the detection of viruses from water is difficult. As described previously, not all the viruses can be detected in only one cell line and the condition in which infection is performed affect the sensitivity of the assay. In addition, the ratio of virions to cell culture infectivity can vary widely. Choi et al. (12) did not find a correlation between the genome concentration of adenovirus via qPCR and plaque assay on A549 cell and HEK 293A cell line. They mentioned that because they did not perform a second passage in all the samples and because plaque assay is not the most sensitive method for the detection of adenovirus some viruses probably remained undetected. Gantzer et al. (21) also failed to find a correlation between the detection of enterovirus genome via RT-PCR and infectious viruses using the BGM cell line. Other studies have found that not all the enteroviruses can be detected using only the BGM cell line (27, 53).

Microorganisms normally present in fresh and seawater play an important role in the inactivation of viruses. Ward et al. (68) investigated the role of microorganisms in the inactivation of viruses in fresh water from different locations. They found that the inactivation rate depended on the type of virus and the location of where the sample was collected. Microorganisms also play an important role in the degradation of naked viral

RNA in seawater (65). In sterile seawater, naked viral RNA can be detected up to 27 days at 24°C after its addition; however, in non-sterile seawater, the naked RNA can be detected for only 3 days at 24°C. Similar results comparing the detection of the infectious poliovirus and its viral genome in seawater have been described (69). In unfiltered seawater, the detection of poliovirus by PCR was related to its detection in cell culture. However, in filtered seawater, poliovirus was detected by PCR for twice as long as in cell culture. Microbial related activity such as RNAses may be responsible for the RNA degradation after viral inactivation in unfiltered seawater. When microorganisms are eliminated in filtered seawater, the degradation of the viral genome is reduced and no relationship is observed between viral inactivation and genome detection by PCR.

In wastewater, only a 90% reduction in poliovirus genome was detected by PCR while a 99% reduction of the infectious virus occurred after 60 days of incubation (59). In treated wastewater, the detection of the enterovirus genome has not been correlated with the isolation of infectious viruses (22). In phosphate buffer, the addition of clay decreases the inactivation rate of coxsackievirus B and degradation of the genome (23). These results may explain the longer survival of enterovirus in wastewater because viral particles tend to attach to solids found in wastewater preventing inactivation of the virus.

DEGRADATION OF THE VIRAL GENOME AND ITS RELATIONSHIP TO VIRAL INFECTIVITY DURING DISINFECTION

The degradation of the viral genome by disinfection can be estimated by determining the concentration or presence of amplifiable genome before and after the exposure. As described previously the sensitivity of this approach depends on the

location and size of the fragment analyzed and is limited to the specific mode of action of the disinfectant evaluated (36, 55). Ma et al. (39) studied the relationship of PCR and cell culture after exposure of poliovirus to different disinfectants. They found that PCR results were comparable to cell culture when assessing the disinfection ability of high levels of chlorine and high pH, because these conditions degrade the nucleic acids. Other disinfectants such as ethanol do not result in nucleic acid degradation suggesting that PCR techniques may not be useful for the assessment infectivity for agents or temperatures that degrade the nucleic acid of the virus (39). For example, the RNA of rotavirus has been demonstrated to remain amplifiable by RT-PCR after exposure to ethanol and drying but was not amplifiable after loss of infectivity by chlorine and peroxide (43). Table 5 is a summary of various studies in which the impact of disinfectants on detection of virus by PCR and cell culture has been compared. It has been reported that the qPCR estimation of amplifiable genome treated with these disinfectants can be correlated with viral inactivation, however, this l results in an underestimation of the inactivation rates (36, 54, 55, 56).

CONCLUSIONS

Different approaches have been used to assess viral infectivity using PCR. One approach is to use damage to the capsid, which results in loss of protection of the nucleic acid or changes in the antigenic properties of the viral capsid to discriminate between infectious and non-infectious viruses. Enzyme pre-treatment and assessment of viral attachment to cell receptors is used in this assessment. Other PCR approaches analyze damages to the nucleic acid that result in the impairment of the PCR reaction include the

long target region (LTR) and 5' non-translated region (NTR) PCR. Because the process of viral replication in the host cell varies with viral type, it is doubtful that any direct PCR method will be totally satisfactory for assessing viral viability. However, the application of these approaches provides a more reliable understanding of the factors that contribute to viral inactivation. Currently, combining PCR and cell culture offers the best approach to assess viral infectivity including the detection of slow growing viruses such as HAV. However, there are difficulties in obtaining a cell culture model for the detection of important waterborne pathogens such as norovirus, leaving the use of direct PCR as the most feasible technique. Presently, the interpretation of PCR results in the detection of viruses in water and assessment of disinfectant efficacy should be on a case-by-case basis considering the type of water, mode of action of the disinfectant and the type of virus.

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TABLE 1. Advantages and disadvantages of the use of cell culture and PCR for the detection of viruses from water.

	Advantages	Disadvantages	Comments	References
Cell culture	Direct measure of infectivity Sample volume (usually between one to 7 mL per flask)	Time required for detection (varies between viruses, usually between 4 to 30 days) Toxicity to compounds from environment (false positive) Does not detect non-culturable viruses and slow growing viruses (non-cytopathic) May require multiple cell lines	Detection by observable cytopathic effect (CPE). Susceptibility between different types of virus varies between cell lines.	(16, 20, 46)
Direct PCR (RT-PCR)	Specificity and sensitivity Can be used for non-culturable virus and non-cytopathic viruses Require less time for detection	Not a direct measure of infectivity Affected by inhibitory compounds found in the environment (false negatives) Low sample volume (usually between 10 to 100 μL	Detection based on the specific amplification of target regions in viral genome In RNA viruses, a reverse transcriptase step is needed before PCR amplification Sensitivity based on the number of viral particles by reaction	(1, 3, 20, 49, 58)

TABLE 2. Modifications of PCR (RT-PCR) used to infer viral infectivity

Approach		Description	Advantage	Disadvantage	References
5'NTR RT-PCR	Targeting the 5' and 3' non coding region of the viral RNA	These regions are more susceptible to degradation, specifically to chlorine and chlorine dioxide which target the secondary structure of this region	The 5' is the most sensitive region of the Picornaviridae genome to degradation	May not work in other viral families which may have different genome organization	(37, 36, 54, 8)
LTR RT-PCT	The use of a long fragment as target for amplification.	It has been found to correlate between the length of the region amplified and the sensitivity of the RT-PCR to detect damage in the genome	Increase the sensitivity of the PCR to detect damage in viral genome. It have been combine successfully with 5'NTR RT-PCR	The amplification of a large fragment may reduce the sensitivity of the PCR detection	(55, 56, 4; 36)
Enzyme treatment pre-RT-PCR	Treatment with proteinase and RNAse before RT-PCR	The proteinase degrades the damaged capsid and then the RNAse degrades the viral RNA. Intact viral capsid may not be degraded by proteinase and may protect the RNA against RNAse	Practical and easy step to incorporate in the assay	Did not detect capsid damage caused by inactivation at 37°C	(41, 42)
Immuno-capture PCR	Antibody captured of the virus for RT-PCR	Damage in the viral capsid may change the antigenic properties of the virus and specific viral antigen-antibody complex may not form	Good for isolating virus from large volume of water and from contaminated sample	Did not have the sensitivity to discriminate between infectious viruses and non infectious viruses	(42, 17, 25, 52)
Viral cell attachment and PCR	Attachment of the virus to a cell monolayer and detection of the attached viruses by PCR	Inactivated viruses did not attach because capsid damage to the cell monolayer therefore yield a negative PCR	A lot of potential in it applications	Further studies are needed	(37)

TABLE 3. Advantage and disadvantages of the use of ICC/PCR and modification for the detection of viruses from water

Approach	Description	Advantages	Disadvantages	Reference:
ICC/PCR	The virus is amplified in the host cell assays and detected by PCR	Toxicity can be identified Faster results than cell culture Larger sample volume Can detect non-cytopathic viruses Less susceptible to inhibition than direct PCR	Do not detect non-culturable viruses In cases of high titer samples can detect the viral genome without any growth in the host cells May require multiple cell lines	(46, 48)
ICC/Strand specific RT-PCR	Specifically detects the viral negative strand in the cells in the case of positive stranded viruses	Indication of infectious virus in high titer samples	Less sensitive than ICC/PCR	(31)
ICC/mRNA RT-PCR	Specifically detects the mRNA of adenovirus in the cells after infection	Indication of infectious viruses in high titer samples Increase in sensitivity	Same as ICC/PCR	(33)

TABLE 4. Comparison of the virus inactivation and degradation of the viral genome in different sources of water

Type of water	Log_{10} reduction in cell culture titer	Viral genome detection (Log_{10} reduction in genome concentration)	Comments	References
River	4.5	3.0	Ratio log N/No infectivity / log N/No genome ~0.6	(57)
Unfiltered seawater	4 in 18 days	Negative after 2 days at 23°C	RNA genome poliovirus RT-PCR targeting 5' NTR	(65)
		Negative after 11 days	Poliovirus initial concentration 10^4 PFU/mL at 22°C	(69)
Filtered seawater	4 in 30 days	Negative after 28 days at 23°C	RNA genome poliovirus RT-PCR targeting 5' NTR	(65)
		Detected after 60 days	Poliovirus initial concentration 10^4 PFU/mL at 22°C	(69)
Bottle water	3.5 in 16 days at 35°C	2 after 145 days at 35°C	Poliovirus initial concentration 10^5 MPN/mL	(24)
Wastewater	2.4	One after 60 days at 25°C	Poliovirus	(59)
Phosphate buffer (pH 7.2)	One in 15 days (not detected after 89 days)	One in 30 days (not detected after 180 days)	coxsackievirus B3 ~10^3 MPN/mL RT-PCR target 5' NTR	(23)
Phosphate buffer (pH 7.2) + Clay[a]	One in19 days (not detected after 94 days)	One in 100 days (not detected after 417 days)		

[a] 200 mg/L Na-montmorillonite
Log_{10} reduction= $Log_{10}(N/N_0)$
N= number of viruses at time T
N_0 = number of viruses at time 0
PFU plaque forming units
MPN most probable number

TABLE 5. Degradation of viral genome after treatment with disinfectants

Disinfectant	Concentration	Exposure (time)	Reduction on infectivity (log_{10} reduction)	Reduction on amplifiable genome (log_{10} reduction)	Virus	References
Chlorine	2500 mg/L	20 min at 24°C	Non-infectious	Not detected	Rotavirus[a]	(38)
	1 mg/L	6 min	4	3	Poliovirus 5' NTR 149 bases	(39)
	10 mg/L	30 min	Non-infectious	Not detected	HAV 5' NTR 1023 bases[b]	(36)
Chlorine dioxide	5 mg/L	25 min		3.5	Poliovirus (5' NTR 6989 bases fragment length)	(55)
		120 min		4	Poliovirus (5' NTR 169 base fragment length)	(37)
	7.5 mg/L	10 min	Not infectious	Not detected	HAV 5'NTR 1023 bases[c]	(37)
Ozone	0.37 mg/L	10 s		3	Norovirus	(54)
		300 s		4.5		
		10 s	7	3	Poliovirus	
		300 s		5		
Hydrogen peroxide	6%	20 min at 24°C	Non-infectious	Not detected	Rotavirus	(43)
UV light		200 μW cm^{-2} for 2.5 hours at 24°C	Non-infectious	Not detected	Rotavirus	(43)
		20 mJ s cm^{-2}	3	One	Poliovirus (1,869 bases fragment)	(56)
		150 mJ s cm^{-2}	7	0.5	Poliovirus (78 bases fragment)	
		22 mJ s cm^{-2}	5	One	Poliovirus (5' NTR 149 bases)	(39)
Ethanol	80%	20 min	Non-infectious	Detected	Rotavirus	(43)

[a] Stock concentration of rotavirus 10^8 PFU/mL
[b] Initial concentration of HAV was $10^{5.75}$ $TCID_{50}$/mL
[c] Initial concentration of HAV was $10^{5.47}$ $TCID_{50}$/mL

APPENDIX B

COMPARISON OF BGM AND PLC/PRC/5 CELL LINES FOR TOTAL CULTURABLE VIRAL ASSAY OF SEWAGE

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ABSTRACT

The objective of this study was to compare PLC/PRF/5 and BGM cell lines for Total Culturable Viral Assay (TCVA) analysis of treated sewage effluents. Samples were collected before and after chlorination from an activated sludge wastewater treatment plant and from the effluent of a high-rate enhanced flocculation system followed by UV light disinfection. Cell monolayers were observed for cytopathic effect (CPE) after two passages of 14 days each. Monolayers exhibiting viral CPE were tested for the presence of adenoviruses and enteroviruses by polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR). Eight percent (8%) of the samples exhibited CPE on BGM cells, and 57% on PLC/PRF/5 cells. Only enteroviruses were detected on the BGM cells, while 30% and 52% of the samples were positive for enteroviruses and adenoviruses, respectively on the PLC/PRF/5 cells. Thirty percent (30%) of the samples were positive for both adenoviruses and enteroviruses in chlorinated activated sludge effluent. Thirty percent (30%) of the samples were positive for adenoviruses in the UV treatment effluent but no enteroviruses were detected. In conclusion, the PLC/PRF/5 cells were more susceptible to enteric viruses than BGM cells. The use of BGM cells for TCVA may underestimate viral concentration in sewage effluent samples. The PLC/PRF/5 cells were more susceptible to adenoviruses, which is important in the evaluation of UV disinfection systems because adenoviruses are highly resistant to UV inactivation.

INTRODUCTION

The detection of enteric viruses requires concentration of large volumes of water and assay on susceptible cell lines (12). The Buffalo Green Monkey Kidney (BGM) cell line is very sensitive to infection by poliovirus and coxsackievirus B and is the most common cell line used for the detection of such viruses in water (4). The BGM cell line recommended for the Total Culturable Viral Assay (TCVA) is the standard method for the enumeration of enteric viruses from water (18). TCVA depends on the ability of the viruses to propagate in BGM cells and create morphological changes or cytopathic effects (CPE) that can be identified and quantified. The combination of the BGM cell line with other cell lines such as A549, human colon adenocarcinoma (Caco-2) and primary liver carcinoma (PLC/PRF/5) for the surveillance of enteric viruses in water has been reported to increase the sensitivity for the detection of adenoviruses, Coxsackie A viruses, echoviruses and astroviruses, or other viruses that do not grow effectively on BGM cells (2; 16, 20).

Primary liver carcinoma (PLC/PRF/5) is a more efficient cell line for the propagation of adenovirus than 293, Chang, KB, and A549 (5). In addition, PLC/PRF/5 cell line has been effectively used for the detection of adenoviruses in river and treated drinking water (19) and it is also susceptible to infection by many enteroviruses, including Coxsackie B viruses and reoviruses (6). The selection of a sensitive cell line for the determination of curturable viruses in water is important because it decreases the cost and efforts required by avoiding the use of multiple cell lines.

The objective of this study was to evaluate the BGM and PLC/PRF/5 cell lines for TCVA in treated sewage samples. Samples from a wastewater treatment plant before and after chlorination, chemically enhanced flocculation effluent, and UV treatment were collected and assayed for TCVA on both BGM and PLC/PRF/5 cell lines.

MATERIALS AND METHODS

Sampling. The South Shore Wastewater treatment plant is an activated sludge wastewater treatment plant (WWTP) located along Lake Michigan in the City of Oak Creek, Wisconsin. The South Shore WWTP is one of the two WWTP in the Milwaukee Metropolitan Sewerage District (MMDS). The MMSD serves 1.8 million clients in 1100 km^2, 5% of the system is combined sewage and storm water collection. Samples were taken from the effluent of the secondary clarifier before and after disinfection with chlorine. High rate chemical flocculation treatment is designed to treat the wet weather plant influent (WWI) during storm events. The two chemically enhanced clarification units used were DensaDeg® (Infilco Degremont, Richmond, VA) and ACTIFLO ® (Krüger, Cary, NC). These units use alum as a coagulant at concentrations between 150 to 250 mg/L. The effluent from these units was subjected to disinfection by exposure to UV light (40,000 $\mu Ws/cm^2$). The samples were collected in the months of May and July of 2005.

Total culturable virus assay. One liter samples were shipped on ice from the WWTP to the laboratory and assay within 48 hours after sampling. The samples were concentrated by organic flocculation as described previously (18). Re-suspended in 30 mL of 0.15 M

sodium phosphate, the final after re-suspension pH was adjusted at 7.2. and 0.3 mL of 100X antibiotic mix containing 10,000 units of Penicillin G, 10,000 units of Streptomycin Sulfate and 25 μg/mL of Amphotericing, and 0.3 mL Kanamycin sulfate (10 mg/mL) and 0.3 ml of Gentamicin (5mg/mL) was added. The concentrates were frozen at -80°C until assay on cell culture. The efficiency of the organic flocculation was determined using poliovirus Type 1 (LSC-2ab, obtained from Baylor College of Medicine) and averaged 60%. The concentrates were split into 15 ml subsample was inoculated in 2.5 ml volume into 75 cm^2 flasks containing cell monolayers and 4.5 mL subsample was inoculate 1 mL volumes into in 25 cm^2 flasks. In the case of toxicity or if all the samples were positive the concentrate was diluted one tenth and 1 mL inoculate into 25 cm^2 flasks. The PLC/PRF/5 cell line (passage lever 26-36, American Type Culture Collection, Manassas, VA; ATCC # CRL-8024) and BGM cell line (passage lever 121-140, Unites States Protection Agency, Cincinnati, OH) were used in this study. The cells were between 3 to 5 days old with a confluent monolayer at the time of infection. The flasks were incubated with the sample at 37°C with slow agitation for two hours. The concentrate was then removed and the flasks were covered with Eagle's maintenance media (Gibco™ Invitrogen Corporation, Grand Island, NY) with 2% fetal bovine serum (Hyclone, Logan, UT) and incubated for 14 days at 37°C. The flasks were examined for CPE every day. The medium was changed every four days. Flask showing signs of CPE were frozen at -20°C and thawed several time, filtered though a 0.22 μm pore size membrane filter and inoculated onto flasks containing fresh cell monolayers (3-5 days old) to confirm viral CPE. After 14 days of incubation, the negative flasks were

frozen and thawed several times and 1 mL of the supernatant was assayed on 25 cm^2 flask containing a fresh cell monolayer (3-5 days old). This second passage was observed over 14 days for CPE. Samples were passed up to three times. All CPE positive flasks were confirmed as described previously. The viral MPN/L was determined using the MPN General Purpose Program by Hurley and Roscoe (7)

One step RT-PCR and nested-PCR for the detection of Enteroviruses. The cell supernatant was obtained after several freeze/thaw cycles as described before, heated to 97°C for five minutes and chilled on ice before the RT-PCR. Ten μL of cell supernatant was mixed with 40 μL of RT-PCR reaction mixture. The reaction mixture contains: 1.0X of reaction buffer (10X buffer: 100 mM of Tris-HCl, pH of 8.00 and 500 mM of KCl), 3.5 mM of $MgCl_2$, 300 μM of each dNTPs, 1.2 μM of Random Hexamers (Applied Biosystems, Roche Molecular Systems Inc. Branchburg, New Jersey), 10 units of RNAase inhibitor (Applied Biosystems, Roche Molecular Systems Inc. Branchburg, NJ), 25 units of MuLV reverse transcriptase (Murine Leukemia virus Reverse Transcriptase, Applied Biosystems, Roche Molecular Systems Inc. Branchburg, NJ), 0.5 μM of each primer P1 and P2, 1.2 units of Amplitaq GOLD (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ) and nuclease free water for a final volume of 50 μL.

The nested PCR mixture consists of: 1X PCR buffer, 2.9 mM of MgCl2, 200 μM of each dNTP, 0.4 μM of each primer (P1 and P33), 1.25 units of Amplitaq GOLD (Applied Biosystems, Roche Molecular Systems Inc. Branchburg, NJ) and 2 μL of the one-step RT-PCR products for a final reaction volume of 50 μL. The primers specific for enteroviruses were: P1 upstream 5' CCT CCG GCC CCT GAA TG 3' and P2

downstream 5' ACC GGA TGG CCA ATC CAA 3' and Ent 33 downstream 5' CCC AAA GTA GTC GGT TCC GC 3' (14). The first round of PCR used the set of primers P1 and P2, with an amplified fragment of 195 bp. The second round of PCR used the set of primers P1 and P33, with an amplified fragment of 105 bp.

The conditions for the RT-PCR were modified from the conditions previously published by Reynolds et al. (13). The PCR condition for the one-step RT-PCR was: 44°C for 60 min, 99°C for 10 min, 25 s at 50°C, 45 s at 72°C, and 35 cycles of 25 s at 94°C, 25 s at 55°C and 45 s at 72°C, and a final extension of 72°C for 7 min. The PCR condition for the second round was: 10 min at 94°C, 25 s at 55°C, 45 s at 72°C, and 35 cycles with 25 s at 94°C, 25 s at 60°C, and 45 s at 72°C, and a final extension step of 7 min at 72°C.

Detection of adenovirus by PCR. The PCR procedure for adenovirus was obtained from Van Heerden et al. (19). The primers for adenovirus were obtained from Avellón et al. (1). The primers for the first round of PCR were ADHEX1F 5'AAC ACC TAY GAS TAC ATG AAC 3' and ADHEX2R 5'KAT GGG GTA RAG CAT GTT 3', with an amplified fragment of 473 bp. The primers for the second round of PCR were ADHEX2F 5' CCC MTT YAA CCA CCA CCG 3'and ADHEX1R 5' ACA TCC TTB CKG AAG TTC CA 3' with an amplified fragment of 168 bp. The PCR mixture consists of: 1X PCR buffer , 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 1 μM each primers, one unit of Amplitaq GOLD polymerase, 10 μL of sample and water to a total volume of 50 μL. The conditions for the first round and nested PCR were the following: 10 minutes at

94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; and a final extension of 10 min at 72°C. The conditions for both PCR reactions were the same.

Quality control assurance. The cell culture facility and incubator are physically separated from the PCR facility. One biological hazard type II hood was used for processing the concentrated samples and another for cell supernatants. All the RT and PCR reagents were mixed in the workstation in the room for reagent preparation. The first round PCR product and nested PCR solution was mixed in a workstation exclusively for this function. The PCR workstations had no forced airflow and UV light. All the areas for the analysis were physically separate. All the reagents were stored in a separate room from the samples. The workstations were cleaned with 10% bleach solution and exposed to UV light for at least 20 min. Different equipment used in each room was not used in other areas (i.e. pipets, tips and lab coats were exclusively used for each room). The PCR thermocyclers are in another room outside the work area. RNA free water was used as a negative control.

Statistical analysis. The reduction in viral concentration was calculated using the following formula: Log10 reduction= log10 (Nt/No). The difference in titer between both cell lines was analyzed using one-way analysis of variance (Minitab 14, Minitab Inc. Cary, NC).

RESULTS

The results of the TCVA are shown in Table 1. Only 3 samples out of 37 (8%) were positive for culturable virus on the BGM cell line. Two samples from secondary

clarified effluent and one sample from the chlorine contact effluent were positive. The maximum MPN value was 1.68 MPN/L. Using PLC/PRF/5 cells, 21 out of 37 (57%) samples were positive for culturable viruses, with an average of 28.5 MPN/L. The highest value obtained was 341 MPN/L. The average TCVA results before chlorination (the secondary clarifier effluent) was 35.8 MPN/L, and the highest value was 135 MPN/L. The average TCVA results after chlorination was 15.7 MPN/L, and the highest value was 34.8 MPN/L. This is a 50% or 0.26 $\log_{10}$ reduction in the concentration of viruses.

After flocculation of the wet-weather influents (WWI), the average TCVA was 75.8 MPN/L and the highest was 341 MPN/L. After UV disinfection this was reduced to 3.6 MPN/L, and the highest value was 15.8 MPN/L. This was a reduction of 1.32 $\log_{10}$.

Eighteen of the samples that were positive using the PLC/PRF/5 cell line were negative using the BGM cell line. No samples were positive on BGM cell line and negative on PLC/PRF/5 cell line. The amount of virus detected on the PLC/PRF/5 cell line TCVA was up to 44 times greater than the values obtained using BGM cells. The difference in titers obtained between the PLC/PRF/5 and BGM cell lines was statistically significant ($p=0.007$).

All the flasks, including monolayers negative for CPE, were tested by PCR for the presence of adenoviruses and enteroviruses to access the presence of non-cytopathic enteroviruses or adenoviruses on the BGM monolayer. None of the samples were positive for adenoviruses and one sample from the secondary clarifier was positive for enteroviruses. All PLC/PRF/5 monolayers showing CPE were confirmed for the presence of adenoviruses and enteroviruses by PCR. Seven samples (33%) were positive

for enteroviruses and 11 samples (52%) were positive for adenoviruses out of 21 samples. Three samples were positive for both viruses. From the 18 positive samples with the PLC/PRF/5 cell line and negative on the BGM cell line, 6 of them were positive for enteroviruses and 8 of them were positive for adenoviruses.

Figure 1a shows the adenoviruses and enteroviruses identified in CPE positive flasks on the PLC/PRF/5 cell line of the WWTP effluent samples. On the pre-chlorination samples (secondary clarifier effluent), the number of positive flasks with adenovirus was 5 and with enteroviruses was 3. In the chlorine contact effluent, adenoviruses and enteroviruses were detected in equal number of samples (3 each).

Figure 1b shows the adenoviruses and enteroviruses identified in CPE positive flasks on the PLC/PRF/5 cell line of the treated WWI effluent samples. The effluent of the chemically enhanced flocculation system was directed to a UV chamber for disinfection prior to discharge. Two samples were positive before UV treatment: the adenoviruses were detected 4 of the CPE positive flasks and enteroviruses in 3 flasks. After UV light treatment, three samples were positive for infectious viruses with one positive flask each: adenoviruses were detected in 1 flask and the other two CPE positive flasks were negative to adenovirus and enterovirus.

DISCUSSION

Our results demonstrated that the PLC/PRF/5 cell line was more sensitive for the detection of culturable viruses than the BGM cell line. Grabow et al. (6) compared the use of four different cell lines including BGM and PLC/PRF/5 for the detection of viruses

from wastewater from different localities in South Africa. They found that the number of samples that produce CPE on PLC/PRF/5 cell line were double the number of samples that produce CPE on BGM cell line, except from one sample in which the water had been impacted by animal contamination.

In our study, no adenoviruses were detected in the BGM cell line. Previous studies have demonstrated the detection of adenovirus on BGM cells from water samples using a combination of cell culture and PCR indicating that CPE was not produced in the BGM cells (10). Because we found a high number of samples positives for adenoviruses producing CPE on PLC/PRF/5, we tested all of the second and third passage BGM flasks by PCR with specific primers for adenoviruses. For the detection of adenovirus genome in cell supernatants by PCR, incubation times of at least 10 days may be needed at concentrations as low as 10^{-2} $TCID_{50}$ in inoculums of concentrated sample used for cell culture assay (10). In our study, each passage was incubated for 14 days, and between two to three passages for each sample, therefore, there was sufficient time for the slower growing adenoviruses to be detected on the BGM cell line. In addition, it has been reported previously that adenoviruses can be detected in cell lines such as A549 by PCR (8) and PLC/PLF/5 by CPE (6) in samples that were negative using the BGM cell line.

A study of the long term enteric viruses surveillance of sewage at Jones Island WWTP, one of the main plants in the Milwaukee Metropolitan Sewerage District, reported that the enteroviruses isolated from sewage included echovirus (80%), coxsackievirus B (14%), poliovirus (4%) and coxsackievirus A (1.1%) (15). Seven different cell lines were used in that study, which included MK-1, HEP-2, HPS, HEL,

BGM, RD, and Caco-2. Fourteen percent of the enteroviruses were isolated on BGM cells (15). Eighty three percent of the coxsackievirus B and 1.9% of the echoviruses were isolated on the BGM cell line (15). Previous studies using clinical isolates have indicated that the BGM cell line is very sensitive to coxsackievirus B and not very sensitive to echovirus infection (3). There is no previous work describing the sensitivity of PLC/PRF/5 cell line to echovirus infection. But the greater detection of enteroviruses observed in comparison with BGM and the proportion of echovirus previously reported in sewage (15), suggest that the PLC/PRF/5 cell line may be more sensitive to echovirus infection than the BGM cell line.

The proportion of enteric viruses previously reported in the sewage from Milwaukee area was 84% for reovirus, 28% for enterovirus and 4% for adenovirus (16). The proportion of viruses producing CPE found in our study was 52% for adenoviruses and 33% for enteroviruses and 15% unidentified. Lee et al. (9) reported the detection of reoviruses in BGM cells that were negative for adenovirus and enterovirus using PCR. Those studies suggest that the unidentified viruses in our study may be reoviruses. In our study, the proportion of adenovirus isolated was higher that in previous studies (16). That may be because of the greater sensitivity of PLC/PRF/5 cells for the detection of adenovirus (5) and seasonal variability of viral concentration reported in sewage (16), taking in consideration that our study was conducted over a period of one month.

We found that the proportion of viruses isolated before disinfection changed in contrast to the proportion of viruses isolated post disinfection. The proportion of enteroviruses increased after disinfection with chlorination, suggesting that chlorine

disinfection produced at this plant during wet weather was not very effective for inactivating the enteroviruses detected in this study. This may be because the free chlorine in sewage combines with ammonia to produce chloramines (which is less effective than chlorine). In contrast, the UV treatment was very effective at inactivating enteroviruses but not adenoviruses. This is in agreement with adenoviruses being very resistant to UV inactivation (11, 17).

The use of multiple cell lines will increase the probability of isolating the maximum number of viruses from wastewater (3, 6). However, this drastically increases the cost per sample, and usually only one cell line is used. Currently, the recommended cell line in the United States for water and wastewater analysis is the BGM cell line. The advantage of using the PLC/PRF/5 cell line for the detection of viruses from treated sewage is not only its greater sensitivity but also that the viruses detected with this cell line were more resistant to disinfection and treatment. If only the BGM cell line had been used then the effectiveness of the disinfectant treatment process would have been overestimated. In conclusion, our results demonstrate that the PLC/PRF/5 cell line was a more suitable cell line for the enumeration of total culturable virus from sewage than the BGM cell line.

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TABLE 1. MPN numbers obtained with BGM and PLC/PRF/5 cell lines and viral genome detected from different treatments

Treatment	BGM cell line		PLC/PRF/5 cell line	
	MPN/L	PCR results	MPN/L	PCR results
Secondary clarifier effluent	0	-[a]	11.8	-
	1.58	E[b]	70	A[c]
	0	-	0	NA[e]
	1.48	-	8.69	AE[d]
	0	-	10.2	A
	0	-	8.24	A
	0	-	15.1	-
	0	-	127	A
	0	-	136	E
	0	-	0	NA
	0	-	6.8	A
Chlorine contact effluent	0	-	11.8	-
	1.63	-	33	A
	0	-	0	NA
	0	-	0	NA
	0	-	28.4	E
	0	-	0	NA
	0	-	24.7	E
	0	-	20.3	-
	0	-	34.8	E
	0	-	19.8	A
	0	-	0	NA

TABLE 1. *continued*

Treatment	BGM cell line		PLC/PRF/5	
	MPN/L	PCR results	MPN/L	PCR results
Enhanced primary treatment	0	-	0	NA
	0	-	0	NA
	0	-	341	AE
	0	-	114	AE
	0	-	0	NA
	0	-	0	NA
UV effluent (primary treated)	0	-	0	NA
	0	-	0	NA
	0	-	7.35	A
	0	-	0	NA
	0	-	0	NA
	0	-	15.8	-
	0	-	9.13	-
	0	-	0	NA
	0	-	0	NA

[a] Negative for adenovirus and enterovirus genome

[b] Positive for the presence of enterovirus genome

[c] Positive for the detection of adenovirus genome

[b] Positive for the detection of adenovirus and enterovirus genome

[e]Not analyzed

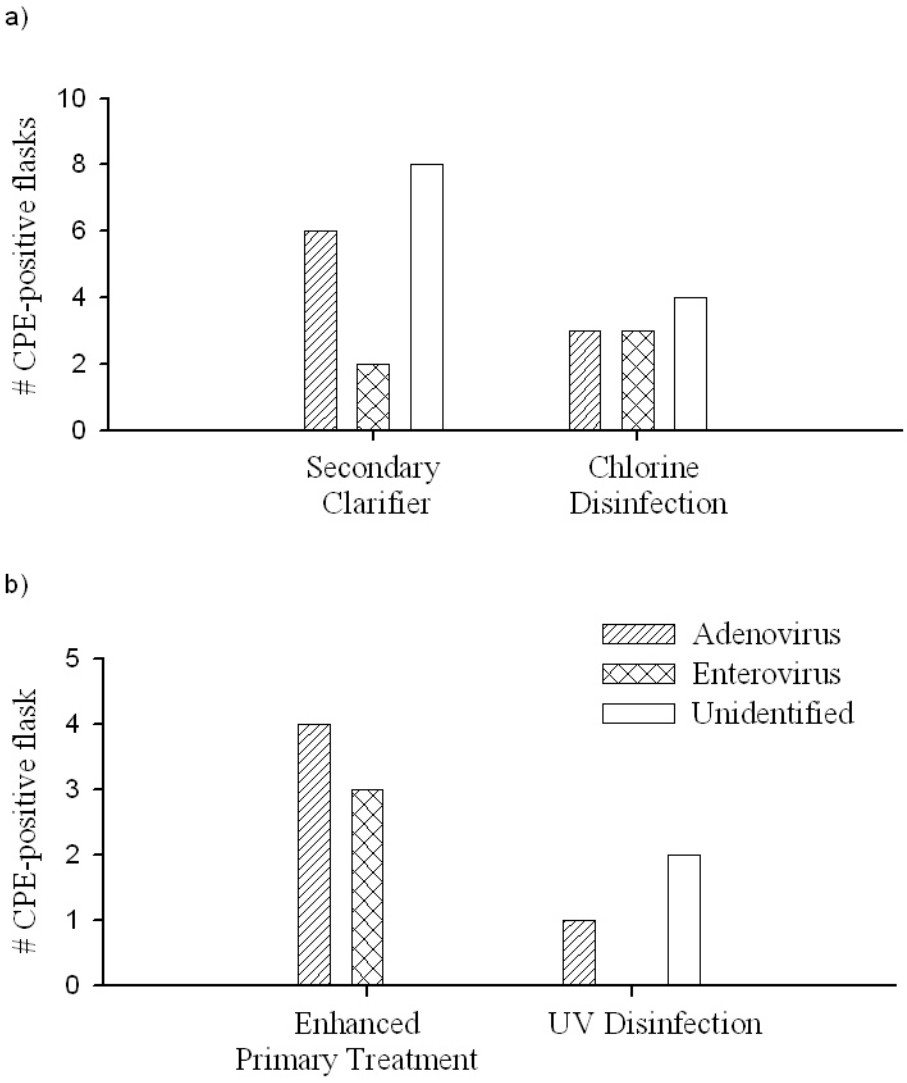

FIG 1. Detection of adenoviruses and enteroviruses in PLC/PRF/5 positive flask from the effluents from (a) secondary clarifier and chlorine disinfection, and from (b) enhanced primary clarification and UV disinfection. Unidentified are flasks that were negative for adenoviruses and enteroviruses but demonstrated viral cytopathic effects.

APPENDIX C

OCCURRENCE OF ADENOVIRUSES AND NOROVIRUSES IN SECONDARY WASTEWATER AND COMBINED SEWAGE OVERFLOWS

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ABSTRACT

The contribution of combined sewer overflows (CSO) to the viral contamination of receiving waters was determined. Adenovirus concentrations were determined using the Primary Liver Carcinoma (PLC/PRF/5) cell line and confirmed by Polymerase Chain Reaction (PCR). Norovirus concentration was determined using the Most Probable Number (MPN) and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). Ninety-three water samples were collected during dry weather and 29 samples were collected during wet weather. CSO events significantly increased the concentration of culturable viruses, adenoviruses and noroviruses in the receiving waters (P=0.001). During dry weather, 73 % of samples were positive for viral cytopathic effect (CPE), adenoviruses were detected in 44 % of the positive cell cultures, and norovirus were detected in 11 % by direct PCR. During wet weather, 100 % of the samples were positive for CPE, 93 % positive for adenoviruses, and 51 % for norovirus. Our results shows CSOs can contribute significant viral loading to receiving waters.

INTRODUCTION

The treatment of wastewater is very important in reducing the occurrence of waterborne infectious diseases. The objective of a wastewater reclamation plant (WRP) is to reduce contamination of water bodies by reducing the nutrients and microbial loads, including human pathogens of the discharged treated sewage (17, 19). In older large cities, sewage is combined with storm water prior to treatment. During wet weather, the amount of combined sewage often surpasses the treatment capacity of the WRP and the combined sewer overflow (CSO) is discharged untreated into the water body.

CSO often result in decreased water quality by increasing the biological oxygen demand as the result of releasing nutrients (5) and decreasing the microbial quality of the receiving waters (4). In addition, CSO's have been found to increase the concentration of microbial indicators of fecal contamination such as fecal coliforms, fecal streptococci, *Clostridium perfringens*, *Aeromonas spp*. and F-RNA bacteriophages in water (7). High-rate flocculation and disinfection treatments have the capacity to treat the combined sewage during wet weather conditions and to reduce the amount of undisinfected CSO. Disinfection treatment for wet weather sewage flows includes the use of ozone and ultraviolet light (UV) irradiation (27, 28).

Adenovirus and norovirus are two of the nine microbiological agents on the USEPA's Contaminant Candidate List (CCL). Adenovirus is a non-enveloped, double-stranded DNA virus. It has been described as the most resistant enteric virus to UV disinfection (23, 24). Adenoviruses have been detected in surface waters (8), source waters (2), and wastewaters (21).

Norovirus is a non-enveloped, positive strand RNA virus. Norovirus is one of the leading agents responsible for viral gastroenteritis in the United States (6). Reverse transcriptase polymerase chain reaction (RT-PCR) has proven to be successful for the detection of norovirus in water (3, 11, 14, 16), however, virus infectivity cannot be ascertained (18).

Information concerning the prevalence and concentration of pathogens in CSOs are necessary for treatment design and risk assessment during wet weather. The concentration of total culturable viruses (TCV), adenoviruses, and noroviruses were analyzed in the receiving waters of three activated sludge WRPs outfall during dry-weather conditions and wet-weather conditions to determine the contribution of CSO in increasing the viral load in the receiving water.

MATERIALS AND METHODS

Sampling. Water was collected from three different wastewater reclamation plants (WRP) outfalls, upstream the outfall and downstream the outfall (Table 1). The treatment of these WRPs consisted of activated sludge secondary treatment with no disinfection. All these plants discharge into a large canal waterway system and do not disinfect treated wastewater because recreational activity is limited to non-contact (i.e boating). Samplings was conducted between the months of June and October for two consecutive years on a monthly basis depending on the weather conditions. Between 120 to 280 L water samples were collected using 1-MDS filters and eluted using beef extract (pH 9.0). Samples were then concentrated by organic flocculation to a final volume of 30 mL. The efficiency of the organic flocculation was determined using poliovirus type 1

(LSc-2ab, obtained from the culture collection of the Department of Virology and Epidemiology at Baylor College of Medicine, Houston, TX) and averaged 60%.

Total culturable viral assay. The concentrates were aliquoted into subsamples of 2.5 mL and used to inoculate 75 cm^2 flasks containing primary liver carcinoma (PLC/PRF/5, American Type Culture Collection, Manassas, VA; ATCC # CRL-8024) cell monolayers; subsamples of one mL and 0.1 mL were used to inoculate 25 cm^2 flasks. The cells were between 3 to 5 days old with a confluent monolayer at the time of inoculation. The flasks were incubated with the concentrate at 37°C with slow agitation for two hours. The concentrate was then removed and the flasks were covered with Eagles maintenance media (Gibco™ Invitrogen Corporation, Grand Island, NY) with 2% bovine serum (Hyclone, Logan, UT) and incubated for 14 days at 37°C. The flasks were checked daily for cytopathic effects (CPE). The media was changed every four days. Flask exhibiting CPE were frozen (-20°C) and thawed several times, filtered though a 0.22 μm pore size membrane filter, and inoculated onto flasks containing fresh PLC/PRF/5 cell monolayers as described previously to confirm viral CPE. After 14 days of incubation, the flasks not demonstrating CPE were frozen and thawed several times, and one mL of the supernatant was assayed on 25 cm^2 flasks containing a fresh cell monolayer. This second passage was also observed for 14 days. Samples were passed to fresh cell monolayers up to three times. The viral Most Probable Number (MPN)/L was determined using the MPN General Purpose Program developed by Hurley and Roscoe (12)

Detection of adenovirus by PCR. The presence of adenovirus in the cell culture was confirmed by PCR (25). The primers for the detection of the hexon capsid protein gene

region of the adenovirus genome were obtained from Avellón et al (1). The PCR mixture consists of: 1X PCR buffer (10X buffer: 100 mM of Tris-HCl, pH of 8.00 and 500 mM of KCl 9, pH 8.00), 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 1 μM each primers, one unit of Amplitaq GOLD (Applied Biosystems, Roche Molecular Systems Inc. Branchburg, NJ), 10 μL of sample and water to a total volume of 50 μL. The conditions for the first round and nested PCR were the following: 10 minutes at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; and a final extension of 10 min at 72°C. The conditions for both PCR reactions were the same.

Viral RNA extraction and RT- PCR for detection of norovirus. The samples were extracted in triplicate using a QIAamp viral RNA extraction kit (Qiagen, Valencia, CA) as described by the manufacturer. A sample concentrate of 140 μL was extracted and the purified RNA was re-suspended in 60 μL. The washing steps were repeated twice as recommended by Qiagen technical support in order to remove inhibitory compounds.

One-step RT-PCR was performed for the detection of the norovirus genome polymerase region A using the primers described by Vinje et al (26). The one-step PCR mixture consists of: 1x PCR buffer (the 10x PCR buffer contains 150 mM Tris-HCl (pH 8.0) 500 mM KCl, Applied Biosystems), 3.5 mM of MgCl, 300 μM of each dNTP, 25 units of Murine Leukemia Virus (MuLV) Reverse Transcriptase (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ), 10 units RNase inhibitor (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ), 2.5 mM of Random Hexamers (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ), 0.5 μM each primer specific for the polymerase region of the norovirus genome (MJV 12 and

Reg A), 1.5 units of Amplitaq gold (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ), 10 μL of sample and water to a total volume of 25 μL. The RT-PCR conditions were the following: 30 min at 42°C, 15 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; and a final extension of 10 min at 72°C. For semi-nested PCR, the followed internal primer was used MP 290: 5'-GAY TAC TCY CS/ideoxyl*/ TGG GAY TC-3'(personal communication with Jean Vinje). The semi-nested PCR mixture consisted of 1x PCR gold buffer, 2.5mM of MgCl, 200 μM of each dNTP, 1 μM of internal primer mp 290 and 1μM of primer Reg A, 1.5 units of Amplitaq Gold, 2 μL of RT-PCR product and water for a total volume of 50 μL. The PCR conditions for the semi-nested PCR were as follows: 5 minutes at 95°C, 40 cycles of 30 s at 94°C, 30 s at 49°C and 30 s at 72°C, and a final extension of 10 min at 72°C.

Samples were assayed in triplicate and diluted 10, 100 and 1000 fold, so that an MPN estimate was obtained for norovirus genomes in each sample.

Quality control assurance. The cell culture facility and incubator are physically separated from the PCR facility. Two PCR workstations with no airflow and UV light were used to provide clean working areas. One biological hazard type II hood was used for processing the samples. All the areas for the analysis were physically separated. All the reagents were stored in a separate room from the samples. The workstations were cleaned with 10% bleach solution and exposed to UV light for at least 20 min before and after use. Equipment used in each room was not transported to other areas (i.e. pipets, tips and lab coats were exclusively used in only one room). The PCR thermocyclers are located in another room outside of the work area. RNA free water was used as a negative

control. All the RT and PCR reagents were mixed in the workstation in the room for reagent preparation.

Statistical analysis. The differences between samples from sampling locations and the impact of CSO were analyzed using analysis of variance (Minitab 14, Minitab Inc., Cary, NC)

RESULTS

Over the two-year study, 122 samples were collected of the impacted waterways. During dry weather, 93 samples were collected with no combined sewage overflow reported (CSO). During the wet weather, 29 samples were collected after reported rain events with CSO discharges. Of the total samples collected, 22 were taken from the outfall, 42 samples were taken upstream (in reference to the outfall) and 58 samples were taken downstream. The results of the samples are presented in Table 2. The CPE positive PLC/PRF/5 flasks were used to calculate total culturable virus (TCV) numbers, and the adenovirus numbers were calculated using the CPE positive flask that were also positive for the confirmation of adenovirus by PCR. Of the 93 samples collected during dry weather, 68 samples were positive for TCV, and of these 41 were positive for human adenoviruses. Ten samples were also positive for human norovirus by direct PCR of the concentrates. All the samples collected after rain demonstrated CPE, and adenoviruses were detected in 27 of the samples. The sensitivity calculated for the detection of CV was 0.1 MPN/100L. Norovirus was detected in 15 of the 29 samples. A significant difference ($P<0.001$) in the concentration of all viruses between the dry and wet weather samples was observed (Table 3). No significant difference in the concentration of viruses

between the waterways of the three WRPs was observed. There was however a significant difference (*P= 0.016*) in norovirus concentration between the samples collected in the outfall, upstream of the outfall and downstream of the outfall (Table 3). However, no significant difference was found in TCV and adenovirus concentrations between these locations. High concentrations of TCV and adenovirus were found during wet weather conditions in the samples collected upstream at the waterways of WRP 2 and 3. Figure 1 compares the concentration of viruses with and without CSO. The average virus concentrations were higher during reported CSO events, including adenoviruses and noroviruses.

The norovirus concentrations in the samples collected in the outfall and downstream were significantly higher than those collected from upstream samples (Figure 2). The equivalent volume tested was calculated to be on average of 400 ml for the RT-PCR detection of noroviruses. The sensitivity for the detection of norovirus by direct RT-PCR was calculated to be 5.8 MPN/100L.

DISCUSSION

This study demonstrates that combined sewer overflows have significant impact on viral concentration in the receiving waters. We found that 73% of the dry weather samples and 100% of the wet weather samples were positive for infectious viruses. The concentration of infectious viruses increased ten fold after wet weather conditions. We also found that only 15% of the dry weather WRP outfall samples were positive for noroviruses, and 7% of the downstream samples. In contrast, in the samples collected after rain events, all the outfall samples and 61% of downstream samples were positive

for norovirus. This increase may be the result of contribution from storm waters which may contain animal pathogens washed from surfaces and untreated sewage (22). Katayama et al. (15) monitored the occurrence of noroviruses and infectious enteroviruses after rain events in the effluent of the waste water treatment plant and in the receiving waters. They reported that 26% of the samples were positive for norovirus and 50 % for infectious enteroviruses and these concentrations remain for 4 days after CSOs events.

Higher concentrations of norovirus have been found in the months of December to January, and correspond with the increase in the number of norovirus cases (9). The samples of this study were collected between the months of June to October, not necessary the peak season for norovirus infections. The concentrations of norovirus found at the outfall of the WRPs ranged from 104 to 114 PCR MPN/100 L, and downstream from the outfall range from 0 to 76 PCR MPN/100L. In studies where the samples were collected during the winter the concentrations of norovirus ranged from 826 to 7499 PCR units/ L in treated sewage, and from 4 to 4900 PCR units/L in the receiving river (20). Therefore, it is possible that if the samples were collected during the winter, the concentration of norovirus would be even higher.

In this study, adenovirus concentration in the outfall of the treated discharge ranged from 45 to 65 MPN/100L during dry weather conditions and from 58 to 401 MPN/100 L after rain events. Concentration of adenovirus in influents of WRPs can range from 0 to 200 infectious units/L of water (21), and 10^7 genomes/100L (10). These concentrations are higher than the concentrations found in this study after rain events,

when CSOs occurs, the released sewage is largely untreated. This may indicate that the concentration of pathogens may be diluted after a rain event because of an increase in the volume of the water and mixing of the effluent with the receiving waters. However, the release untreated sewage still results in increased adenovirus concentrations in the receiving waters.

The upstream samples were used as a reference to estimate the impact of the WRP outflow on water quality. After wet-weather, high concentrations of TCV and adenovirus were found, which resulted in the lack of statistical difference between the samples collected in the outfall, downstream and upstream of the outfall. For example, during dry weather, 48% of the samples collected at the WRP outfall, 42% collected downstream and 25 % collected upstream were positive to adenovirus. In the samples taken after rain events, 100% of the samples collected at the outfall, 94% of the samples at the downstream and 75% of the samples collected at the upstream were positive for adenoviruses. The higher concentration of virus found in the upstream samples during rain events may result because of the increase in the volume of water released from the outfalls, which may impact water flows in the canals. This impact was more prominent in the samples collected from the Stickney WRP (WRP 3) which has a capacity to treat 1.2 billion gallons per day. However, a statistical difference in the concentration of norovirus between sampling locations was found. This statistical difference for norovirus suggests that it may be a better indicator of CSO and sewage effluent quality than adenovirus. Human adenovirus has not been detected from any other source other than humans (13), and has been also suggested as a possible candidate for an indicator of the

presence of human fecal contamination in water (8). The higher concentration of adenovirus in the upstream samples in comparison with norovirus is difficult to explain but may be because more volume of sample was analyzed in the detection of adenovirus by cell culture, which results in greater sensitivity than the sensitivity of the detection of norovirus by semi-nested RT-PCR. In conclusion, combined sewage overflows contribute a significant viral load to the receiving waters.

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TABLE 1. Description of Wastewater Reclamation Plants (WRP) and sampling locations in the waterways

WRP	Capacity (MGD)	Sampling location (approximate distance from the outfall in feet)	
		Downstream	Upstream
Calumet (#1)	338	6,600	5,500
North Side (#2)	330	2,800	2,500
Stickney (#3)	1,200	6,300	8,200

The distance from the outfall was calculated to be at least 15 waterways widths

TABLE 2. Viruses concentration: dry versus wet weather

		Total Culturable viruses				Adenovirus				Norovirus			
WRP		Dry weather		Wet weather		Dry Weather		Wet weather		Dry weather		Wet weather	
1	UPSTREAM[c]	4/12[a]	5[b]	1/1	14.7	2/12	0.3	1/1	11.0	0/12	0	0/1	0
	OUTFALL	4/7	68	1/1	117	3/7	45	1/1	58	1/7	103.9	1/1	651
	DOWNSTREAM	10/18	101	4/4	1053	7/18	63	4/4	561	0/18	0	1/4	21.3
2	UPSTREAM	4/10	0.6	3/3	1009	3/10	0.4	2/3	751	0/10	0	0/3	0
	OUTFALL	4/5	87			1/5	53			1/5	284		
	DOWNSTREAM	10/10	11	10/10	873	3/10	7.3	9/10	438	0/10	0	7/10	501
3	UPSTREAM	8/12	21	4/4	569	4/12	3	3/4	338	3/12	34	1/4	288
	OUTFALL	6/7	218	2/2	848	5/7	66	2/2	401	1/7	114	2/2	4145
	DOWNSTREAM	10/12	47	4/4	321	7/12	28	4/4	211	3/12	76.4	3/4	829
All		68/93	55	29/29	682	41/93	28	27/29	385	10/93	44.7	15/29	578

[a] positive samples/total of samples.

[b] Average MPN/ 100L

[c] Location of the sample in reference to the WRP outfall.

TABLE 3. *P* value obtained from the analysis of variance

Effect	Viruses detected		
	Culturable viruses	Adenovirus	Norovirus
Wet weather event[a]	***<0.001***	***<0.001***	***<0.001***
WRP[b]	*0.8*	*0.766*	*0.181*
Sampling location[c]	*0.367*	*0.644*	***0.016***

The *P* values are for [a] the effect of the combined sewage overflow (CSO) event, [b] the wastewater treatment plant (WRP), and [c] the sampling location (outfall, downstream, upstream) on the viral numbers.

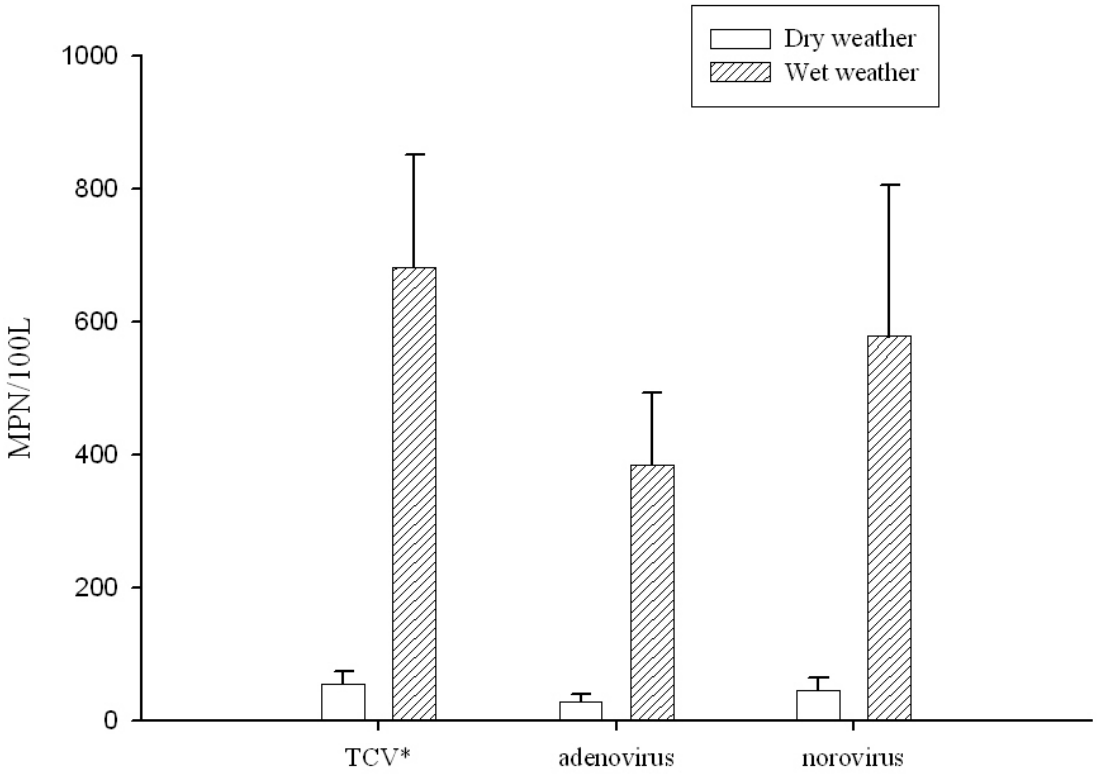

FIG 1. Effect on rainfall events in the concentration of viruses in the receiving waters.
* total culturable viruses

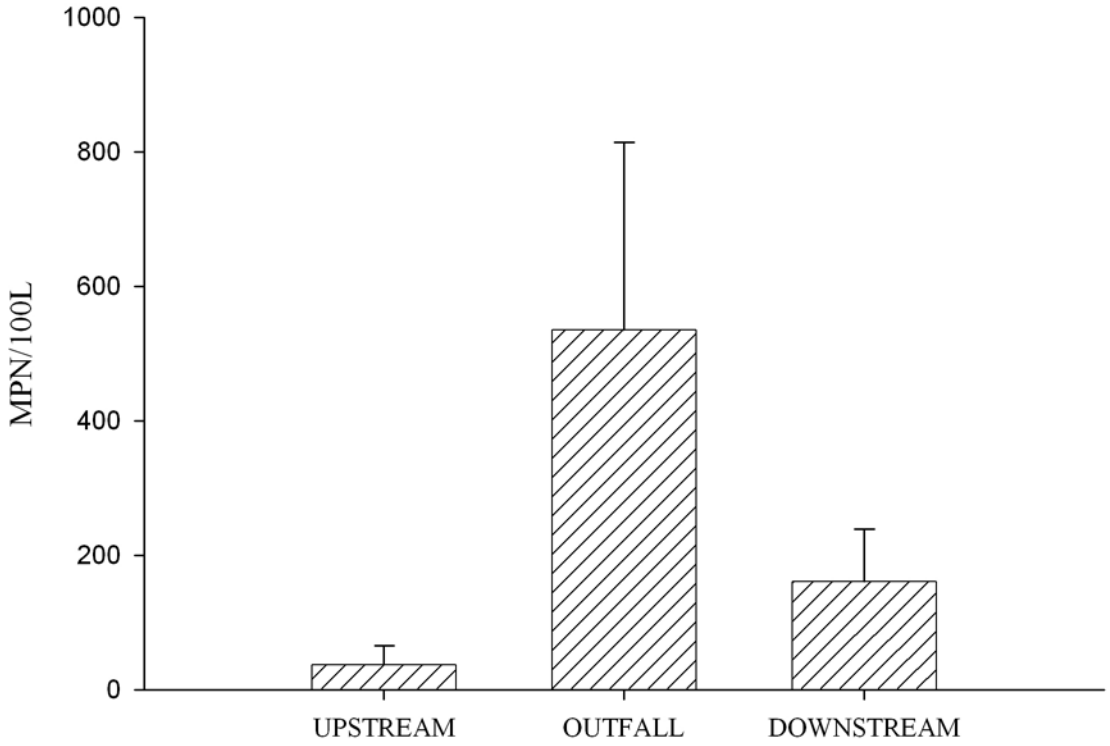

FIG 2. Average concentrations of norovirus in relationship to outfall