ABSTRACT

Norwalk virus (NV) is an important cause of waterborne gastroenteritis, yet its response to UV disinfection is unknown. Non-culturable and without an animal model, reverse-transcription PCR (RT-PCR) or an alternative nucleic acid amplification method is the only practical way to detect and quantify this virus in water. However, we have found that RT-PCR underestimates the loss of virus infectivity by UV radiation. In an effort to improve the predictability of RT-PCR amplification for the loss of virus infectivity by UV disinfection, we investigated alternatives to single amplicon, direct RT-PCR. In addition to NV, coliphage MS2 as well as poliovirus type 3 (PV3) and type 1 (PV1) were included in the study to compare infectivity assays to RT-PCR assays for quantifying virus inactivation by UV radiation. Based on infectivity assays, a UV dose of 150 mW-sec/cm^2 reduced MS2 and PV1 by 4.8 and $\geq$5.8 $\log_{10}$, respectively. By direct RT-PCR amplification of genomic targets of about 200-250 BP, this dose of UV radiation produced from zero to 1 $\log_{10}$ titer reduction in any of the three viruses. In order to improve the detection of UV damage to viral genomic RNA, RT-PCR amplification was performed at multiple targets. However, even at a high dose of 550 mW-sec/cm^2, there was no increase in the observed reduction in virus titer by RT-PCR amplification of 200-250 BP targets. In an effort to

recover and detect only antigenically functional virions containing genomic RNA, antigen capture/RT-PCR was applied to UV irradiated viruses. No significant decrease in AC/RT-PCR titer was observed for all three viruses, despite extensive inactivation of the infectivity of MS2 and PV1. Amplicon length was increased to 500-1300 BP in an effort to improve detection of UV damage. The increase in amplicon size better estimated PV1 inactivation and detected greater inactivation at lower doses for NV. The results of this study indicate that RT-PCR amplification at multiple genomic sites and antigen-capture RT-PCR using polyclonal antibodies are not reliable methods to quantify the inactivation of viruses by UV radiation. However, amplification of longer RNA genomic targets may improve the ability of RT-PCR to predict reduction of virus infectivity by UV radiation.

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UV Disinfection of Norwalk Virus and Other Enteric Viruses and Their Detection by RT-PCR

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1.0 INTRODUCTION

Management of drinking water microbial quality and disinfection efficacy is currently dependent on the measurement of concentrations or frequency of occurrence of indicator organisms, primarily enteric bacteria, as a surrogate parameter of fecal contamination. Human enteric viruses are a significant cause of waterborne disease; however, they are rarely measured in water because their detection by conventional cell culture assay is limited by its technical difficulty, cost and low sensitivity. Enteric viruses generally have a greater resistance to conventional chlorine disinfection and are frequently detected in finished water meeting the current coliform bacteria standards. Although conventional virus detection methods are time consuming, expensive, and slow to produce results, the recent Information Collection Regulation (ICR) requires that these methods be utilized in the monitoring of surface sources of drinking water on an interim basis. Recent advances in molecular techniques capable of rapid and sensitive detection of viral nucleic acids may provide an improved method to monitor important viral pathogens in water.

Responsible for up to 23% of waterborne acute gastroenteritis in the U.S. (Kaplan, 1982), Norwalk virus (NV) and related viruses must be addressed in the management of drinking water quality. However, the lack of suitable laboratory techniques has limited the ability to detect NV in water or determine its removal and inactivation by water treatment

processes. Reverse transcription PCR (RT-PCR) offers a rapid and sensitive method to detect NV and other enteric viruses. However, a significant limitation of RT-PCR is its inability to detect infectious virus particles. The relationship between RT-PCR amplification of viral nucleic acid and the presence of infectious viral pathogens has been poorly characterized

UV radiation is a promising disinfection technology for drinking water and other waters, especially with increased concern about chlorination by-products. UV radiation appears to effectively inactivate a variety of microorganisms without generating toxic residuals and shows promise as a disinfection alternative to chlorine. The microbial effect of UV at 254 nm is believed to be due to damage of the microbial genome by the formation of pyrimidine dimers, hydrates and adducts. While UV damage to most viruses, particularly single-stranded viruses such as Norwalk virus, is irreversible, an important question is whether damage to the nucleic acid, and consequently the loss of viral infectivity, can be correlated to a reduced RT-PCR amplification signal.

The purpose of this study is to determine the UV inactivation of Norwalk virus and, for comparison, poliovirus and MS2, a male-specific RNA coliphage. Virus reduction, as determined by RT-PCR endpoint titration was compared to virus reduction by cell infectivity assay. However, RT-PCR may detect the nucleic acid of non-infectious as well as infectious viruses. Several alternative approaches to RT-PCR were

investigated in an effort to improve RT-PCR detection of infectious virus. These alternative approaches included antibody capture RT-PCR (AC/RT-PCR) and direct RT-PCR (D/RT-PCR) amplification of multiple short targets as well as amplification of relatively long genomic targets.

2.0 OBJECTIVE

The objective of this investigation was the development of an improved RT-PCR method which would enable the detection of UV inactivated virus, particularly virus such as Norwalk virus which are currently without infectivity assays.

3.0 LITERATURE REVIEW

3.1 Disinfection

Application of disinfection processes is an important barrier against pathogenic microorganisms in the production of finished water for public consumption and the treatment of wastewater effluent for release to the environment. In 1908, the first continuous application of chlorine was utilized to treat a municipal water supply (Craun, 1986; Dychdala, 1983). The routine use of chlorine disinfection and the introduction of water filtration have drastically reduced the number of epidemics caused by waterborne infectious diseases (Craun, 1988).

Disinfection is utilized both by drinking water and wastewater treatment plants to produce finished water and effluent of specific microbial quality. In drinking water, primary disinfection achieves the desired pathogen kill, with the secondary disinfection providing a stable residual in the finished water to prevent regrowth of microbial pathogens in the distribution system. In the case of wastewater, disinfection is the most important stage in the processing of pathogen-containing wastewater such that it is suitable for release to surface waters or, as water becomes an increasingly limited resource, wastewater reuse.

As a strong oxidant, chlorine is an effective bactericide and removes unwanted color, reduced iron and manganese. Chlorine is also relatively inexpensive, easy to apply and useful both as a primary and

secondary disinfectant. However, in 1974 Rook identified chloroform as a by-product of chlorine disinfection. Evidence of possible adverse health effects of trihalomethanes (THMs) and other disinfection by-products (DBPs) as well as growing concern about the environmental impact of toxic residuals in treatment plant effluents (Ward and DeGraeve, 1978), motivates research into the efficacy and impact of alternative disinfectants, including ultraviolet radiation.

3.2 Disinfection Kinetics

In 1908, Hariette Chick described exponential disinfection kinetics based on the correlation between the rate of inactivation of anthrax spores by chlorine and known chemical reaction kinetics. In this equation, now known as Chick's law (Chick,1908), the number of organisms destroyed per unit time is proportional to the number of organisms remaining:

$$dN/dt = -kN$$

In this equation, N is the number of survivors, dN/dt is the rate of kill, and k is a constant, varying with the characteristics and concentration of the disinfectant. Integration of this equation produces the exponential kill curve:

$$N/N_0 = e^{-kt.} \quad \text{or} \quad \ln N/N_0 = -kt.$$

This equation describes a first-order "single-hit" reaction that assumes the concentration of disinfectant is essentially constant with time. The rate constant, k, is dependent on chemical dose, type of disinfectant,

temperature, pH and characteristics of the target organism. Chick's equation assumes a homogeneous pathogen population.

In the same year, Watson extended this equation to describe the relationship between disinfectant concentration and the required contact time to produce a certain inactivation. This equation is now known as the Chick-Watson model:

$$dN/dt = -kC^nN$$

In this equation, N is the microbial concentration, C is the disinfectant residual, t is the time, k and n are kinetic parameters. In this equation (Watson, 1908), Watson describes the Ct concept, a fundamental tool in the disinfection regulations currently set by EPA. Current standards are set by the EPA in the Surface Water Treatment Rule (SWTR) Guidance Manual and are tabulated by disinfectant type, pH and temperature. Water treatment utilities must meet pathogen removal-inactivation regulations by satisfying the appropriate Ct conditions.

3.3 Factors in Drinking Water and Wastewater Disinfection

Water treatment plants utilize both primary and secondary disinfectants in the production of finished water. Primary disinfection achieves the target level of microbial inactivation or kill. In the case of drinking water, a secondary chemical disinfectant is applied to maintain a residual concentration in finished water to prevent regrowth of pathogenic microorganisms in the distribution system and to protect against post-

treatment contamination from other sources. Factors such as pathogen occurrence, size and other constitutive characteristics, resistance to disinfectant, host specificity, infectious dose, and water or wastewater quality must be considered in the selection and application of the disinfection method.

The diversity of viral, bacterial and protozoan pathogens that must be controlled by drinking water and wastewater disinfection complicates disinfection practice. Enteric viruses in particular show large variability in their sensitivities to disinfectants. Liu *et al.* (1995) investigated the time needed for 4 log inactivation with 0.5 mg/l free chlorine. There was over a $\log_{10}$ variation, ranging from 36.5 minutes for poliovirus, type 2 to 2.7 minutes for reovirus 1. Similar variations in disinfectant sensitivities are seen in bacteria (Butterfield, 1948) and protozoan (Chang, 1944).

Water sources vary in the pathogens they contain and consequently differ in their treatment requirements. According to the Safe Drinking Water Act, water utilities serving greater than 25 customers may soon be required to disinfect groundwater, while surface water sources must be processed both by disinfection and filtration. Contaminated groundwater has been consistently responsible for more waterborne outbreaks than contaminated surface water (Craun, 1990). In the period between 1971-85, contaminated, untreated and inadequately disinfected ground water was responsible for 49% of the waterborne outbreaks. Surface water, untreated, contaminated or inadequately disinfected, was

responsible for 24% of waterborne outbreaks (Craun, 1990). Turbidity, pH, temperature, maintenance of disinfectant residual, disinfectant concentration and contact time are important parameters which must be monitored by treatment plant operators to achieve adequate disinfection of water and wastewater.

3.4 Ultraviolet Radiation

Sunlight has been utilized for centuries as a traditional form of drinking water disinfection. In the early 1900's, UV radiation was used to treat municipal water supplies (Oliver and Carey, 1976). However, due to economic and technological advantages, chlorination became the disinfection method of choice. With increased concern about chlorine generated disinfection by-products and new UV technology, including the production of new, higher intensity, medium pressure UV lamps, UV disinfection is gaining support as an economic and efficient alternative disinfection method (Scheible and Bassiel, 1981; Qualls *et al.* 1989).

UV radiation is highly effective for inactivation of viruses and bacteria and has several advantages over chlorine and other chemical disinfectants. UV disinfection units are readily available, produce no toxic residual, are relatively simple to operate and maintain, require little space and require short contact times relative to chemical disinfectants (Venosa, 1983). These characteristics make UV an attractive choice for groundwater sources of drinking water as well as wastewater treatment and reuse, particularly if used in conjunction with a secondary disinfectant.

Currently, 600 municipal wastewater treatment plants utilize UV as a disinfectant process (Blatchley, 1996). Typically, UV radiation is used in smaller treatment facilities due to the complexities of larger systems that require thousands of lamps. This may change as new, higher intensity UV lamps become available. These new, medium-pressure ultraviolet lamps produce a higher intensity of UV radiation across a broader spectrum. This capacity produces a better UV disinfection capacity per lamp, increases the cost-efficiency of UV disinfection and may provide effective UV inactivation of protozoan cysts.

3.5 Mechanism of UV Inactivation

UV disinfection involves a radiation energy, transmitting energy to atoms or molecules by excitation rather than ionization. As opposed to corpuscular radiation, electromagnetic wave radiation travels at a constant velocity, varying wavelength and frequency inversely to produce the constant velocity of 186,000 miles per second. Although, ultraviolet radiation travels as an electromagnetic wave, it behaves as if a discrete quanta of energy. The marked photochemical and photobiological properties of ultraviolet radiation are a consequence of its relatively short wavelength, high frequency and, consequently, the large-energy of UV quanta.

Discovered in 1801 by J.W. Ritter, UV radiation was first observed as a dark region beyond the visible-light spectrum (Jagger, 1967). Studies

such as those by Gates (1929) and Sykes (1965) separated ultraviolet radiation into different wavelengths of monochromatic light and identified the UV spectrum of primary bactericidal importance as from 240 to 280 nm. The UV action spectra suggests that the primary germicidal effect of UV radiation is caused by nucleic acid damage (Lehman and Olson, 1983; Venosa, 1983; Rogers, 1985).

UV inactivation is proportional to the UV dose and has been shown to follow Chick's Law (Luckiesh and Holladay, 1944) possibly through formation of pyrimidine dimers, and to a lesser extent, pyrimidine hydrates, pyrimidine-thymine adducts and UV induction of single-strand breaks (Witkin,1976; Harm, 1980; Venosa, 1983). Pyrimidine dimers are formed primarily between adjacent thymine residuals on the same DNA strand. Rahan (1973) found that UV irradiation of DNA forms photoproducts that disrupt the DNA structure. The sugar phosphate moiety of nucleic acid does not significantly absorb UV above 220 nm (Jagger, 1967).

Less information exists on the photochemistry of ribonucleic acid. It has been noted that pyrimidine hydrates and uracil dimers are formed in irradiated RNA. Both of these products can cause UV inactivation of RNA. Other pyrimidine photoproducts have also been observed, although no clear correlation to biological inactivation has been determined.

3.6 Factors Affecting UV Treatment

UV irradiation as a method of water and wastewater disinfection has several limitations. With no chemical residual, a secondary disinfectant is necessary to prevent regrowth within the distribution system and protect against post-treatment contamination. In addition, current UV disinfection technology has shown limited inactivation capacity against *Giardia* and *Cryptospordium* cysts at doses typically used for water and wastewater disinfection. UV transmittance, and consequently it germicidal efficiency, is diminished by turbidity, suspended organic and inorganic material. However, UV disinfection of wastewater secondary effluent is gaining support because total kill or inactivation of pathogens is not required and it is unnecessary to maintain an antimicrobial chemical residual (Oliver and Carey, 1976; Wolfe, 1990). In addition, a new method of UV disinfection, pulsed-UV light, has been shown to deliver intense pulses of broad spectrum UV radiation such that a dose of 1000 mW-sec/cm^2 is delivered in a fraction of a second and will reduce *Cryptospordium parvum* oocyst viability by 3-6 $\log_{10}$ (Dunn, 1997). Several factors must be considered when utilizing UV irradiation to inactivate of pathogens in water and wastewater, and these factors are considered in the next sections of this report.

3.6a Method of Production

Germicidal lamps are the most common source of disinfecting UV irradiation. These lamps generate UV radiation by creating an electron flow between electrodes through a low-pressure mercury vapor. The

electronic arc excites mercury atoms, causing collisions with the release of photons in the form of UV radiation. In common fluorescent lamps, the lamp is coated with a phosphor compound that converts the UV radiation to visible light. The glass used in the conventional fluorescent bulb filters out almost all UV. In contrast, the germicidal lamp is not coated with phosphor, and instead utilizes a special glass that transmits all UV. Approximately 85-95% of the UV radiation generated is at a wavelength of 253.7 nm (U.S. EPA, 1986). The temperature and air current surrounding the mercury lamps strongly effects the efficiency of the germicidal lamp. Most commercial lamps are designed to operate at ambient temperature and care must be taken to ensure that the UV lamp bank does not overheat. The lamp intensity will also decrease rapidly with use. Consequently, UV lamps must be carefully monitored and regularly cleaned to remove dust and other substances that may decrease the transmitted UV intensity.

3.6b Water Quality

Penetration of UV energy through water and therefore its germicidal efficiency are affected by several water quality parameters. Water turbidity, iron salts and dissolved organic material are all factors that alter the UV demand of water and wastewater. Turbidity, resulting from suspended solids, may result in the scattering of UV radiation. Suspended solids have been shown to partially protect some bacteria and viruses from UV inactivation (Hoff, 1978; Oliver and Cosgrove, 1977). In

the case of UV disinfection, this protection is likely due to shadowing of pathogens by particulate matter. Dissolved organic and inorganic compounds will absorb UV radiation and consequently decrease the efficiency of UV inactivation. These water quality parameters affect UV transmission in water and must be considered in determination of the dose necessary for disinfection (Harris, 1987; Nagy, 1955; Luckiesh, 1944).

The efficacy of UV inactivation is increased by stirring. This increase in inactivation is a consequence of more effective exposure to UV radiation and, consequently, an increased net UV dosage (Cortelyou, 1954; Budowsky, 1981). In water and wastewater treatment, flocculation and filtration prior to UV irradiation has been shown to increase disinfection efficiency by decreasing UV demand, decreasing shadowing of microorganisms by particulate matter and increasing the dose received by pathogens (Dizer *et al.*, 1993). The treatment plant must set its hydraulic flow in accordance to water quality parameters and the necessary UV dose for appropriate pathogen inactivation (Severine et al., 1984).

3.6c Reactivation

Photoreactivation has been shown to occur when UV damaged cells are exposed to visible light of a wavelength between 300-500 nm, depending on the organism (Jagger, 1958). Light repair was identified by Kelner in 1949 and involves the activation of a photoenzymatic repair process, involving a single enzyme that monomerizes pyrimidine dimers.

Photoreactivation has been observed in phages, however, single-stranded DNA phage are substantially less photoreactivable than double-stranded DNA phage, most likely due to a smaller fraction of nucleic acid damage in the form of pyrimidine dimers. This photoactivated enzyme system acts exclusively on UV-irradiated DNA, with no impact on RNA phage or animal viruses (Harm, 1980).

In addition to light repair, host-cell reactivation and excision repair has been shown to occur in viruses. Both of these repair mechanisms rely on double-strandedness of the nucleic acid molecule. Excision repair enzymes remove pyrimidine dimers from one UV-damaged strand after which resynthesis of the nucleotide sequence is performed using information on the complementary DNA or RNA strand. These repair mechanisms have not be observed extensively in UV inactivated viruses, most likely due to their lack of metabolic machinery to execute this repair (Setlow in Florkin; Stotz, 1967). Yet in consideration of these repair mechanisms, particularly with respect to bacterial pathogens, UV-treated water should not be exposed to visible light during storage (Knudson, 1985).

Multiplicity reactivation is an additional method of repair that can occur in UV-exposed viruses. In this process, the successful infection of a host cell is achieved by complementation of several damaged virions. This reactivation process may be a significant factor with aggregated virus particles. However, multiplicity reactivation is primarily observed in

double-stranded DNA phage and animal viruses. This reactivation process depends on genetic recombination and consequently its effect is small, if not absent, in single-stranded DNA phage (Harm, 1980). However, multiplicity reactivation has been reported to have a small effect in type 1 poliovirus, a single stranded RNA virus (Harm, 1980).

3.6d Virus Aggregates

Aggregation of viruses has been observed both in water and wastewater (Young and Sharp, 1975). Viruses such as poliovirus are known to have increased resistance to disinfection when in an aggregated state (Floyd, 1976; Young, 1975) and as a consequence, complex deviations from first-order inactivation kinetics can occur. Factors such pH, particle concentration, virus type, solvent ionic composition and strength influence the extent of virus aggregation (Sharp, 1976).

3.7 Waterborne Diseases

Hepatitis A virus, *Vibrio cholerae*, poliovirus, *Salmonella typhi*, *Cryptosporidium parvum*, *Giardia lamblia* and Norwalk virus are examples of important human pathogens transmissible by water. With application of disinfection to community water supplies, waterborne epidemics have dramatically decreased in the 20th century. Yet despite improvements in water sanitation, waterborne outbreaks continue to occur. The protozoans, *Cryptosporidium parvum* and *Giardia lamblia* are much more resistant to chemical disinfection than bacteria pathogens such as *S. typhi*

and *V. Cholerae* and continue to present difficulties in modern water supplies. Unless care is taken to control disinfection efficacy with proper filtration and continuous application of effective disinfectant to surface and ground waters, there will continue to be outbreaks of waterborne infectious diseases, including giardiasis, cryptosporidosis, viral hepatitis and viral gastroenteritis.

While there are many historical references to drinking water precautions, it was John Snow's correlation between London's 1854 cholera epidemic and the Broad Street pump water supply which provided the first scientific evidence that an unseen, water-transmissible element could be directly responsible for a disease outbreak. The identification of the etiologic agents of waterborne disease outbreaks continues to be extremely problematic. Many outbreaks are not identified because epidemiological methods lack the sensitivity to detect low-level, waterborne pathogen transmission. In addition, waterborne viral outbreaks due to enteroviruses and other enteric viruses are difficult to detect as assay techniques are technically difficult, time-consuming and costly.

During the period 1986-1994, 130 waterborne outbreaks and 452,964 cases of illness were reported to CDC and the EPA (MMWR CDC Surveillance Summaries, vol 39, 40, 42, 45). Increased surveillance and identification of the etiologic agent(s) of waterborne outbreaks are helping determine whether current regulations and treatment processes are

effective at preventing of waterborne outbreaks due to traditional and emerging pathogens. Provision of drinking water of adequate quality and sufficient quantity continues to be an essential element in efforts to control and protect the public from infectious diseases. While the difficulties in achieving this goal are greater in developing than in developed countries, endemic and epidemic waterborne disease still occur in developed countries, including the United States.

3.8 Drinking Water Regulation

In 1986, Amendments to the Safe Drinking Water Act (SDWA) mandated that EPA establish disinfection requirements to control waterborne pathogens by drinking water treatment, minimize formation of disinfection by-products, establish regulations for maximum contaminant levels (MCLs) for disinfection by-products (DBPs) and prevent discharge of toxic effluent into the environment. The 1996 Safe Drinking Water Act (SDWA) amendments require USEPA to designate deadlines for the regulation of high-risk microbial contaminants and disinfection by-product. By November 1998, EPA must promulgate a stage I D/DBP Rule and an interim Enhanced SWTR (ESWTR). With some delay, the final Information Collection Rule (ICR) was established in May 1996. Surveillance data collected for the ICR will be used to develop an improved, stage II D/DBP Rule and finalize an ESWTR. All municipal water supplies will be required to disinfect source groundwater. Additional

filtration processes will be required for source water influenced by surface water. Water utilities will be monitored to ensure that they meet the difficult task of meeting MCLs for DBPs while maintaining microbial safety standards.

EPA will set separate disinfection criteria for ground waters than those established for surface waters. The Surface Water Treatment Rule treats Giardia and enteric viruses as the most restrictive pathogens as these are the organisms most resistant to disinfection. However, revisions of the SWTR now being developed recognize *Cryptospordium parvum* as an even more resistant pathogen than Giardia or enteric viruses. In recognition of this risk, the MCL of *Cryptospordium* is expected to be set at zero (Pontius, 1997).

The Groundwater Treatment Rule (GWTR) has not yet been developed; however, the draft GWDR targets enteric viruses as the most important pathogens. Enteric viruses are considered restrictive pathogens in groundwater regulation because of their small size, occurrence in groundwater and frequent role in ground water-borne outbreaks. In situations in which *Cryptosporidium parvum* and *Giardia lamblia* are not considered a great concern in drinking water sources, such as with groundwater, UV radiation may be the most suitable alternative disinfection processes.

3.9 Enteric Viruses

Enteric viruses multiply in the alimentary tract, particularly in the lower intestine. Approximately 140 types of enteric viruses can be found in water and wastewater (Bitton, 1994). Fecally shed in concentrations as high as 10^{10} infectious units per gram, unusually stable in environmental waters (White and Fenner, 1986) and generally more resistant to chlorine disinfection than bacterial pathogens, enteric viruses pose a significant public health risk in drinking water.

Norwalk virus, rotavirus, hepatitis A virus, adenovirus and enterovirus infections cause significant waterborne diseases (Blacklow, 1991). Most enteric viruses are non-enveloped and typically have a short incubation period of 7-14 days. Viral gastroenteritis is the second most common viral illness with symptoms ranging from diarrhea, nausea, vomiting, fever, headache, myalgia and malaise. These viruses cause infections which are usually self-limiting but which may be fatal in the very young, elderly, malnourished or immuno-compromised.

3.9a Norwalk Virus

Norwalk virus (NV) is a 26-to 35-nm, nonenveloped, 7.7 kb, positive-sense, single-stranded RNA virus, recently classified on the basis of genome sequencing as a member of the *Caliciviridae* family (Jiang, 1990). With a buoyant density of 1.33 to 1.41 g/cm^3 in CsCl, these viruses lack distinctive morphology when viewed by scanning electron microscopy. There are three open reading frames (ORFs) in the NV genome. The first and longest, ORF 1, is predicted to be initiated by an

internal AUG start codon and to encode a polyprotein precursor to a nonstructural protein. The second ORF is thought to encode a capsid protein that self-assembles into virus-like particles. ORF 3 is at the 3' end of the NV genome and is thought to encode a small protein of unknown function.

The Norwalk group of viruses is a major worldwide cause of epidemic, waterborne gastroenteritis. CDC has estimated that 42% of non-bacterial gastroenteritis is associated with Norwalk virus (Kaplan, 1982). NV is also the major cause of acute gastroenteritis following ingestion of raw shellfish (Gouvea, 1994; Kohn, 1995). Fifty to seventy percent of the U.S. adult population shows serum antibodies to these viruses, suggesting prior NV exposure.

Symptoms of NV infection include mild to moderate nausea (79%), diarrhea (66%), vomiting (69%), headaches, myalgia and malaise. Illness lasts approximately 72 hours with fecal shedding of infectious virus occurring from onset. Spread through fecally contaminated water and shellfish, NV transport, persistence and disinfection kinetics have not been adequately investigated because NV cannot be cultivated in vitro and has no known animal model. Traditionally, diagnosis has been achieved through immune electron microscopy (IEM), radio-immune assay (RIA) and enzyme-immune assay (EIA). These assay techniques are limited by the scarcity of reagents derived from human volunteers and the lack of sensitivity, specificity and standardization. Cloning and molecular

detection techniques such as RT-PCR are increasing the ability of researchers to diagnose and study Norwalk virus and related human calicivirus.

Currently there is evidence of three Norwalk-like virus genogroups, and at least five distinct serotypes. Norwalk, Snow Mountain and Sapporo viruses are the protypes of genogroups 1, 2, and 3, respectively. Norwalk, Hawaii, Snow Mountain, Taunton and the Sapporo strain are currently considered the five prototypes of human calciviruses HuCVs with others strains still being investigated. Additional viruses such as *minireoviruses*, recovered from pediatric patients with nosocomial gastroentritis (Lew, 1994), appear to have genomic organization similar to the Norwalk virus and are likely to soon be classified in the *Caliciviridae* family.

Immunity to NV is not well understood. There are two forms of immunity to NV: short- and long-term. Short-term resistance is serotype-specific (Wyatt, 1974). Long-term immunity deviates from traditional immunity as seen in response to infection with most other viruses. Adults with blood serum antibodies can still be susceptible to reinfection while, in contrast, those without NV serum antibodies are often resistant to infection when challenged. It is thought that genetically determined variation in virus receptors in the intestinal tract is responsible for long-term resistance in the absence of host NV serum antibodies (Baron, 1984; Blacklow & Cukor, 1982; Cukor & Blacklow, 1984). The lack of immunity in the presence of host NV antibodies is less well understood.

3.9b Poliovirus

Throughout recorded human history, paralytic poliovirus cases have been identified. In the mid-1700s, a syndrome identifiable as paralytic poliomyelitis began to be mentioned in the medical literature. (Melnick, 1996), with large epidemics occurring through the first half of the 20th century. With an aggressive poliovirus vaccination program, much effort is currently devoted to the elimination of wild-type poliovirus worldwide.

Poliovirus was classified in 1955 as an Enterovirus (von Magnus, 1955). A subgroup of the family *Picornaviridae*, the genus Enterovirus was formed in response to a large group of viruses similar in their small size (~ 27 nm), an RNA genome, resistance to ether and mild acid (pH 3.0) and multiplication within the enteric tract . This grouping has been validated through modern molecular techniques which have identified sequence homology, similar structure and mode of replication.

There is a large ratio of unapparent poliovirus infections for each paralytic case. Exposure of a non-vaccinated individual to poliovirus may result in a range of responses including assymtomatic infection, mild illness, non-paralytic poliomyelitis and paralytic poliomyelitis. The most common symptoms of mild poliovirus infection include fever, malaise, drowsiness, headache, nausea, vomiting, constipation and a sore throat. In about 1-2% of cases, there is mild muscle weakness with almost always complete recovery. Paralytic poliomyelitis is the most serious

manifestation of poliovirus infection and occurs in 1% or fewer of all cases. Typically the virus does not multiply in muscle, with peripheral muscle and nerve damage secondary to the primary destruction of nerve cells within the central nervous system. In these cases, flaccid paralysis is the most common symptom, caused by lower motor neuron damage. The amount of damage varies and recovery usually occurs within 6 months, with some cases suffering permanent residual paralysis.

The three serotypes of poliovirus share some surface antigens, (Melnick, 1996) but are distinguished by epitope differences clustered primarily on VP1, one of four structural proteins. Propagation of poliovirus in cell culture has allowed not only better understanding of the viruses and reliable diagnosis of infection but also production of a live, oral polio vaccine (OPV). In areas where live OPV is used, vaccine strain poliovirus is often found in sewage and fecally contaminated water. However, other enteroviruses are also found in water and sewage, especially during recurrent annual periods of high prevalence in the population.

3.10 Indicators of Water Quality

Indicator organisms are utilized as easily detectable and quantifiable indicators of fecal contamination, treatment efficiency and reliability of the distribution system (Olivieri, 1983). Disinfection of drinking water and treated sewage, although often effective at reduction of bacterial pathogens, does not necessarily achieve inactivation of viruses.

Consequently, viruses may be detected in waters meeting fecal coliform and other bacterial indicator standards. (Geldenhuys and Pretorius, 1989; Payment, 1985) Better indicator organisms must be identified to protect the public from health risks due to viral contamination. Because direct detection of pathogenic bacteria, viruses and protozoan cysts is often too costly, time-consuming or technically difficult for routine use, indicator organisms are necessary tools in the assessment and monitoring of drinking water, wastewater and recreational water for microbial quality.

Goyal (1983) and Elliott and Colwell (1985) have proposed the following criteria for indicator organisms:

1. **Applicable to all types of water.**
2. **Present in sewage and fecally contaminated water when pathogens are present.**
3. **Numbers correlate with amount of fecal contamination.**
4. **Present in greater numbers than pathogens.**
5. **No aftergrowth or regrowth in water or the environment.**
6. **Survival/persistence greater than or at least equal to pathogens in natural environments and treatment processes.**
7. **Absent from non-fecally contaminated water.**
8. **Easily detected and quantified.**
9. **Constant characteristics.**
10. **Harmless to humans and other animals.**
11. **Numbers in water associated with risks of enteric illness for consumers.**

At present, no indicator organism has met all criteria of an ideal indicator. Yet indicators continue to play an important role in the monitoring and surveillance of water quality.

3.11 MS2 Bacteriophage

Bacteriophage have been extensively studied as potential indicators of pollution, including pollution by waterborne human viral pathogens. These viruses are easily detectable and some studies suggest that certain bacteriophage have comparable survival times to human viruses in environmental waters and water treatment (Havelaar, 1991). Bacteriophage are viruses which infect bacterial hosts, contain DNA or RNA genetic material, and like all viruses, are obligate parasites requiring a specific host for infection, replication, maturation and release. An important feature in the classification of bacteriophage is the site that these viruses utilize to initiate bacterial infection. Those bacteriophages that attach to and infect through pili are called F male-specific phage in comparison to the somatic bacteriophage that adsorb to and infect through the host's membrane.

Havelaar *et al.*, (1993) suggests F-specific RNA bacteriophage are adequate indicators of enteric viruses in fresh water. MS2 is a prototypical F male-specific coliphage. In addition serotyping male-specific RNA coliphage may provide information to differentiate between human and animal contamination sources (Furuse, 1987).

MS2 is useful as a model organism for UV inactivation research. As a F male-specific RNA coliphage, MS2 has single-stranded RNA. With a first-order survival curve for UV inactivation (Harm, 1980; Mattern *et al.*, 1965) and without host-cell reactivation mechanisms, MS2 is a simple and effective bioassay organism (Qualls *et al.*, 1983). The structure and size

of MS2 is superficially similar to human enteroviruses, such as poliovirus as well as human caliciviruses and hepatitis A virus (Havelaar, 1990).

3.12 Methods for Virus Detection

As previously noted, human enteric viruses have been detected in water meeting coliform standards (Melnick, 1980; Berg, 1973). No indicator organism is without limitations and, as of yet, no generally accepted and proven indicator organism directly reflects the presence and concentrations of enteric viruses. Conventional methods to detect human enteric viral pathogens are unable or inefficient at direct detection of low levels of the more important viral pathogens. Recovery and identification methods are limited in their sensitivity, can be time-consuming, expensive, and require skilled labor and advanced laboratory equipment. Some enteric viruses, such as hepatitis A and E viruses, rotaviruses, caliciviruses and enteric adenovirus, cannot be detected at all or with high sensitivity in cell culture. Fast, reliable, sensitive, efficient and economical detection of enteric viruses is an important area of research and development that is essential to the regulation, control and prevention of waterborne disease.

3.12a Conventional Detection Methods

Conventional methods for detection of human enteric viruses include immune electron microscopy, solid phase immuno-assays or cell culture infectivity assays. Conventional detection of pathogenic viruses in

drinking and environmental waters involves collecting a representational sample, concentrating the viruses, extracting viruses from the concentrate, and isolating and quantifying the viruses by cell culture assays. Cell culture assays routinely underestimate virus presence for several reasons. Concentration methods are often inefficient and their efficiency is highly variable. In addition, cytotoxic contaminants are concentrated with the viruses and may interfere with cell assay. Finally, many important pathogenic enteric viruses such as hepatitis A virus, rotavirus and Norwalk virus grow poorly or not at all in cell culture. As a consequence of these limitations to conventional methods, new molecular detection methods for viral nucleic acids are being considered as an alternative approach to effectively and rapidly monitor enteric viruses in water and wastewater.

3.12b Molecular Detection of Viral Nucleic Acid

Molecular cloning of enterovirus and other pathogenic virus genomes enabled the development of hybridization detection techniques (Cova, 1988, Jansen, 1985). However, the low sensitivity of these techniques limits the detection of low levels of enteric viruses in drinking water and environmental waters. With the development of *in-vitro* amplification of specific nucleic acid sequences the detection of low concentrations of waterborne pathogens dramatically improved. The application of *in vitro* enzymatic amplification by PCR, followed by detection of the specific nucleic acid product by oligoprobe hybridization, has provided the sensitivity and specificity required to monitor fecal contamination of water

and other samples (Abbaszadegan, 1993).

Molecular detection of viral pathogens in water samples is limited by conventional concentration protocols which co-concentrate PCR inhibitory substances. Techniques such as guanidinium extraction, (Shieh, 1995) have been developed to purify viral nucleic acid from the concentrate. However, these techniques isolate purified viral nucleic acid, complicating efforts to detect only infectious viral particles. New concentration techniques such as PEG precipitation and antibody-capture PCR (AC/PCR) attempt to concentrate intact virions (Schwab, 1996). In general, PCR results cannot yet be reliably interpreted in terms of detection of infectious or viable pathogens (Maier, 1995; Josephson, 1993). Improved molecular methods to detect waterborne pathogens at low concentrations and in complex environmental samples will strengthen the detection and study of important pathogens. Ultimately, this research will provide better surveillance data of waterborne pathogens, a better understanding of disinfection efficacy and, consequently, enable an improved prevention and control strategy for waterborne diseases.

With recent legislation to regulate disinfection by-products, ultraviolet radiation is gaining support as an alternative disinfection technology, particularly for wastewater effluent and smaller drinking water supplies. Given that many of the more important viral pathogens are difficult or impossible to detect by conventional infectivity assays, PCR or an alternative molecular technique is essential in the study and detection of

viral pollution in water. With the mechanism of UV inactivation occurring at the level of the nucleic acid, it is possible that failure of RT-PCR amplification, as detected by a loss of amplicon signal, may be interpretable as a loss of viral infectivity. Successful adaptation of the sensitive and specific detection of viral pathogens by RT-PCR such that only potentially viable and infectious viruses are amplified would greatly improve the detection of viral pathogens and provide a new basis to determine and possible regulate the virological quality of water.

4.0 MATERIALS AND METHODS

4.1 Virus and Host Cells:

4.1a Norwalk Virus (NV)

Norwalk virus (strain 8FIIa) was processed from composite human volunteer stool samples. A 10% stool slurry in phosphate buffered saline (PBS) was kept at 4°C for 2 hours and the NV was purified by fluorocarbon extraction by vortexing one part organic solvent and two parts stool suspension for at least 2 minutes and then centrifuging at 5000 x g for 20 minutes. The NV extraction process was repeated 6 times using successive volumes of PBS. The aqueous supernatant was recovered and saved as NV stock and frozen at -70°C.

4.1b Poliovirus

Poliovirus type 1 (PV1), strain LSc, and poliovirus type 3 (PV3), Sabine strain, were propagated and assayed for infectivity using continuous cell cultures. PV1 was grown and assayed in the Buffalo Green Monkey Kidney (BGMK) continuous cell line. PV3 was grown and assayed in RD cells, a human embryonal rhabdomyosarcoma cell line. Host cells were grown and maintained in Eagle's Minimum Essential Medium supplemented with 0.015 M HEPES buffer, kanamycin (100μg/mL), gentamicin (50 μg/mL), non-essential amino acids, sodium bicarbonate (0.075%), L-glutamine, 0.04M magnesium chloride, and fetal calf serum (2% for maintenance and plaque assay media; 10% for growth

media). Infected cell lysate was frozen and thawed 3 times and harvested by fluorocarbon extraction as described in previous section. Infectivity data was collected by cell culture plaque assay techniques using 1% neutral red vital dye as has been previously described (Sobsey et al., 1978).

4.1c Coliphage MS2

Coliphage MS2 (ATCC 15597-B1) was propagated and assayed in *E. coli* C-3000 (ATCC 15597) by double (top) agar layer (DAL) plaque technique (Adams, 1959). MS2 stock was harvested from the top agar of confluent lysis DAL plates and suspended in small volumes of PBS. Cell debris was removed by chloroform extraction and centrifugation at 5000 x g for 15 minutes at 4°C. MS2 was recovered in the aqueous phase and stored at -40°C.

4.2 Virus Preparation

Stock virus suspensions were filtered to remove virus aggregates by filtering first through a 0.2 μm-pore-size followed by filtering through a 0.08μm-pore-size, pretreated Nucleopore polycarbonate filter. Pretreatment was performed by passing 3.0 mL of autoclaved, filtered (0.2 μm Millex, Millipore) 0.1% Tween 80 through filters followed by 3 washes with 10 mL of autoclaved, filtered (0.2 μm Millex, Millipore), distilled, deionized water. MS2 was immediately suspended in PBS to minimize aggregation. All monodispersed viruses were stored at 4°C to preserve

infectivity and dispersion. UV irradiation experiments were performed within 48 hours.

4.3 Experimental Procedure

4.3a UV Dosing

Four 25 watt germicidal lamps (Aquifine 3084) produced 254 nm UV radiation which was collimated to provide only radiation perpendicular or nearly perpendicular to the surface of the virus suspension in 60x15mm plastic petri dish. Filtered virus suspension in 10 mL of PBS, gave a liquid depth 0.5 cm, and it was constantly stirred at a slow speed by magnetic mixing during UV exposure. UV lamps were turned on and allowed to stabilize for 10-15 minutes. UV intensity of germicidal lamps was measured with a UVX Digital Radiometer. Exposure times were controlled by blocking out the UV radiation with cardboard before and after calculated exposure times. The irradiated virus suspension was sampled as a function of time and samples were stored immediately on melting ice and assayed for infectivity within 6 hours.

4.3b Dose Measurements

Average intensity (I_{avg}) was determined from an equation first discussed by Morowitz (1950) for the irradiation of stirred suspensions:

$$I_{avg} = I_o \times (1-e^{-aL})/aL$$

Where I_o is the incident intensity, L is the depth of the suspension or path length (cm) and a is the absorbance per cm. Because the turbidity of the

virus suspension, as measured at 254 nm with a Spectronic 1201 spectrophotometer (Milton Roy Company, Rochester, NY), was low, no corrections for scattering were necessary. Dose was computed as the product of known radiation intensity and exposure time (seconds) and could be calculated from:

Dose = Intensity x time

This equation is derived from the Bunsen-Roscoe reciprocity law of photochemistry which states that a constant photochemical reaction occurs if the product of intensity and exposure time is constant (Gates, 1929). The necessary exposure time was calculated by dividing the desired UV dose by the known UV intensity. Samples of the virus suspension were removed at specified intervals, including a nonirradiated sample at time zero. Virus suspensions at each dose were diluted serially 10-fold in 0.1X PCR buffer (Perkin Elmer-Roche, Alameda, CA) and titered the same day in cell cultures by plaque assay. The same virus dilutions were tested as 10 μl aliquots by D/RT-PCR and alternative RT-PCR techniques. Viral inactivation was defined either as is a $\log_{10}$ loss of virus infectivity ($-\log_{10}$ (N_t/N_0)) by plaque assay or as a endpoint titration assay as determined by a loss in RT-PCR amplicon signal.

4.4 Reverse-transcription and Enzymatic Amplification

4.4a Short Target RT-PCR

The RNA-PCR kit from Perkin Elmer-Roche (Alameda, CA) was used

according to the manufacturer's protocol, except the reaction volume for reverse transcription (RT) was increased to 30μl to accommodate a 10μl sample. Random hexamers or 3' gene-specific primers were used in the RT reaction.

Viral RNA was heat-released by adding reaction tubes containing PCR buffer, magnesium chloride, nucleotides, primer and sample to a thermocycler (model 480, Perkin Elmer, Norwalk, CN) preheated to 99°C, for 5 minutes. Tubes were chilled quickly on ice for 1 minute, centrifuged and 50 units of MuLv reverse transcriptase and 20 units of RNase inhibitor were added. After vortexing, tubes were added to a thermocycler preheated to 42°C for 60 minutes, followed by a 5 minute 99°C step to inactivate the reverse transcriptase. After chilling tubes to 4°C and a brief centrifugation, tubes were supplemented with a complete primer set, PCR buffer, magnesium chloride, water and 5 units *Taq* DNA polymerase to a final volume of 100μl. Each reaction tube was overlaid with 80 μL of mineral oil. Tubes were added to the thermocycler preheated to 95°C and programmed for the following PCR profile: 40 cycles of 95° for 1.5 min. (denaturation), 55° for 1.5 min. (primer annealing), and 72° for 1.5 minutes (elongation).

4.4b Long Target RT-PCR

Amplification of long viral genome targets was done using a Titan One Tube RT-PCR System from Boehringer Mannheim (Indianapolis, IN). Utilizing a heat stable AMV Reverse Transcriptase, synthesis of cDNA

was performed at 50°C rather than 42°C. The higher transcription temperature improved reverse transcription of problem genomic regions by reducing the secondary structure of viral RNA. PCR amplification of transcribed cDNA was achieved without additional manipulation of reaction tube to add DNA polymerase activity. A combination of two high fidelity, RNase H- and heat stable DNA polymerases (*Taq* DNA polymerase and *Pwo* DNA polymerase), provided a sensitive one tube, one step RT-PCR system. Addition of DTT, DMSO and EDTA stabilized the template and increased the activity of the enzymes. This single step protocol minimized the opportunity for contamination, reduced handling time, and increased specificity and sensitivity to low copy number by providing opportunity for an increased optimal primer-annealing temperature.

The manufacturer's protocol was followed with a 10 μl sample. Primers, nucleotides, DTT and PCR-grade water were combined in single reaction cocktail and distributed to reaction tubes. The sample was added and the viral RNA heat-released by adding tubes to the thermocycler preheated to 99°C for 5 minutes. After being chilled on ice for 2 minutes, tubes were centrifuged and a second reagent cocktail was added, containing RNase Inhibitor, PCR buffer and enzyme mix. After the addition of a mineral oil overlay to prevent evaporation, tubes were added to preheated thermocycler for a 30 minute RT step at 50°C, followed immediately by 10 cycles of PCR amplification with denaturation at 94°C

for 30 sec., annealing at 60°C for 30 sec. and elongation at 68°C for 1 minute. This thermocycler profile was linked to a similar profile, with the same denaturation and annealing temperatures and times, but with a 5 second extension per cycle to the 68°C elongation step for 25 cycles. The final thermocycler profile was a prolonged elongation step of 7 minutes at 68°C.

4.4c Antibody (Immunoaffinity) Capture RT-PCR

Viruses were captured by antibodies that were bound to a paramagnetic bead. The RNA of captured viruses was heat-released and amplified by RT-PCR. Goat anti-human or goat anti-rabbit immunoglobin G (IgG) antibodies covalently linked to paramagnetic beads were from Bio Mag Advanced Magnetics, Inc. (Cambridge, Mass.) Non-specific binding sites were blocked with 1% bovine serum albumin (BSA)(Boehringer Mannheim, Indianapolis, IN) and the bead-antibody complex was washed twice with 0.05% Tween 20 in PBS. Pooled human serum immunoglobulin (HSIG, GAMMAR, Armour Pharmaceutical Co., Kankakee, IL), diluted 1:200 in 0.1% BSA in PBS, was added and the reaction gently agitated and allowed to incubate for 30 minutes at room temperature. The bead complex was washed twice with 0.05% Tween 20 in PBS and the sample was added. The mixture was incubated for 2-4 hours at room temperature and the antigen-antibody complex was washed five times with 0.05% Tween 20 in PBS. The beads were concentrated with a magnet and resuspended in 50 μl 0.1% PCR buffer. Viral RNA was

heat-released by adding to the thermocycler preheated to 99°C for 5 minutes. The magnetic beads were removed by microcentrifugation for 2 minutes at 10K, the RNA containing supernatant containing the released viral RNA was analyzed immediately by RT-PCR.

4.5 Oligonucleotide Primers for RT-PCR

4.5a Short Target Primer Pairs

Poliovirus, type 1 and type 3 were detected by the pan-enterovirus primer pair described by De Leon *et al.*, 1990. This primer pair amplifies a region from nucleic acid base number 449 to 644 in the 5' non-translated region on the reference enterovirus of coxsackievirus B3 (Chapman et al., 1990). Three primer pairs were used in the amplification of NV short targets. Primer pair p35/p36 was described by Atmar et al, 1995 and amplify a 469 base fragment (4487-4956) inside the polymerase region of the Norwalk virus genome. The NV pol primer pair amplifies a 260 base sequence within the NV polymerase region (4601-4860) and was described by De Leon *et al.*, (1992). The third NV short fragment primer pair, described by Jiang *et al.*, (1993) was designated the IP primer pair, amplified a 224 bp region (2215-2438) at the 5' end of NV ORF 1. There were two MS2 short target primer pairs both selected by Battigelli *et al.*, (1990). Rep 1 and Rep 2 amplify fragments of 234 and 233 nucleotides, (1982-2215, 2298-2330, respectively) with both amplicons within the MS2 replicase region.

4.5b Long Target Primer Pairs

Primer pairs were selected to amplify long NV, MS2 and PV1 genomic targets. Complete viral genomic sequences were located from GenBank using computer software package ENTREZ (Release 24.0, Bethesda, MD). Primer sequences were identified from the literature and appropriate sequences located in target regions were analyzed. The primer length was increased to 28-33 bases to increase the optimum primer pair annealing temperature to approximately 60°C. In addition primer pairs were analyzed for homology and secondary structure. Once appropriate candidate primer pairs were identified, oligonucleotides were ordered from the Nucleic Acids Core Facility (Lineberger Comprehensive Cancer Center, Chapel Hill, NC) and resuspended in PCR-grade water. Primer pairs were tested for sensitivity and the best primer pair chosen for long target amplification.

There are two long target primer pairs for Norwalk virus: NV Amplicon 1 (NV2A/B) is within the 5' non-coding region and is 1346 bp long and NV Amplicon 2 (NVC3/5) is within the polymerase region and amplifies a 922 bp amplicon. The MS2 long target primer pairs amplifies a 494 bp amplicon within the replicase region. The poliovirus polC/D primer pair amplifies a 970 bp amplicon (6116-6981) within the polymerase region.

4.5c Primer Pair Sensitivity

The sensitivity of the primer pairs detection of target virus by RT-PCR was determined for PV and MS2 by amplifying target viruses in samples

serially diluted 10-fold in 0.1% PCR buffer. Standardized 10µl volumes of each dilution were evaluated by RT-PCR and compared with infectivity data available on the same dilution series by plaque assay. The RT-PCR titer was represented as PCR units (PCRU/mL) and was calculated from the assumption of 1 PCRU at the lowest dilution for which an amplicon signal was detected. For PV and MS2, RT-PCR titer can be compared to the cell culture infectivity titer to determine the relative sensitivity of RT-PCR amplification to infectivity (PFU/mL) results. In the case of NV, primer pair sensitivity was compared to relative to the PCRU detection of the most sensitive short target primer pair, the pol primer set (1 1PCRU). Primer detection limits are presented below in Table 4.1.

Table 4.1: Primers for NV, PV and MS2 Amplification by RT-PCR

Virus Primers	Genomic nucleotides	Sequence (5' to 3')	Amplicon Size (bp)	Sensitivity
PV pan-entero 3' 5'	 627-644 449-465	 ACCGGATGGCCAATCCAA (-) CCTCCGGCCCCTGAATG (+)	197	0.01-0.1 PFU D/RT-PCR 10-100 PFU AC/RT-PCR
PV polC/D 3' 5'	 6951-6981 6116-6143	 GGTAGGAAGCAATTACATCATCACCATAGGC (-) CGATCCCAGGCTTAAGACAGACTTTGAG (+)	970	0.1-1.0 PFU D/RT-PCR
MS2 Rep 1 3' 5'	 2195-2215 1982-2001	 GCTTGTTCAGCGAACTTCTTG (-) TAAGCTACGGGAGGCGAATG (+)	234	1.0-10 PFU D/RT-PCR 10-100 PFU AC/RT-PCR
MS2 Rep 2 3' 5'	 2310-2331 2098-2117	 CCCTACAACGAGCCTAAATTC (-) GCAACCTCCTCTCTGGCTAC (+)	233	1.0-10 PFU D/RT-PCR 10-100 PFU AC/RT-PCR
MS2 BREP3/5 3' 5'	 2310-2331 1838-1858	 TCCCTACAACGAGCCTAAATTC (-) ATGAGGATTACCCATGTCGAAG (+)	494	10 PFU
NV Pol 3' 5'	 4840-4860 4601-4621	 GAGAAATATGACATGGATTG (-) CAAATTATGACAGAATCCTTC (+)	260	1 PCRU [a] D/RT-PCR 100-1000 PCRU [a] AC/RT-PCR
NV IP 3' 5'	 2419-2438 2215-2233	 AGCCTGATAGAGCATTCTTT (-) CACCACCATAAACAGGCTGT (+)	224	1 PCRU [a] D/RT-PCR 100-1000 PCRU [a] AC/RT-PCR
NV 35/36 3' 5'	 4936-4956 4487-4505	 CTTGTTGGTTTGAGGCCATAT (-) ATAAAAGTTGGCATGAACA (+)	470	10 PCRU [a] D/RT-PCR 100-1000 PCRU [a] AC/RT-PCR
NV 2A/B 3' 5'	 1938-1970 624-656	 TGGTGATGACTATAGCATCAGACACAAATTGCA (-) ATGCTATATACATAGGTCAAGGCAAGACGGTGG (+)	1347	0.1 PCRU [a]
NV C3/C5 3' 5'	 4907-4931 4010-4038	 TGAGAATTTGAGTGAGGCGGGCTGG (-) GGCCCATCCCTACAACAGGTACTACGTGA (+)	922	0.1 PCRU [a]

[a] RT-PCR NV Amplicon Sensitivity of Expressed Relative to D/RT-PCR Amplification of pol primer region (1PCRU)

4.6 Analysis of Viral Amplicons by Agarose Gel Electrophoresis

Amplified PCR products were analyzed on 1.2 % agarose gels for long target amplicons and on 1.7% agarose gels for short target amplicons. Agarose gels were prepared by dissolving the agarose in 1X TAE buffer (0.04 M Tris base, 0.005 M sodium acetate, 0.001 M EDTA) using a microwave oven for heating. Agarose gels were supplemented with ethidium bromide and PCR samples were suspended in loading buffer and injected into pre-formed wells in the agarose gel. Gels were electrophoresed and DNA products were visualized by UV transillumination (Ultra-Lum, Paramount, CA) with a 100 bp or 1 Kb DNA ladder (GibcoBRL, Gaithersberg, MD) utilized as a PCR product length reference.

4.7 Data Analysis

Inactivation, particularly of single-stranded, monodispersed viruses such as the NV, MS2 and PV used in this study can typically be characterized by first-order kinetics and modeled by Chick's law. The survival ratio (N_t/ N_0), where N_0 is the initial titer and N_t is the virus plaque count at time t (time of exposure), relates the virus concentration at sampling point t (relating to a particular dose) to the concentration of viruses at the start of the experiment. The survival ratios were log-transformed and plotted as a function of UV dose. The survival data for MS2 and PV was calculated from cell infectivity data and expressed as a ratio of the mean plaque forming units per mL (PFU/mL) at each sampling point relative to the mean virus concentration at time zero. The UV dose is directly proportional to the time of UV exposure per the Bunsen-Roscoe reciprocity law and the survival ratio can therefore be treated as linear function of the

UV dose.

RT-PCR amplification was also used to estimate virus loss due to UV irradiation. The loss of RT-PCR amplicon signal was treated as a measure of UV inactivation. The greatest 10-fold dilution giving a RT-PCR signal was assumed to contain 1 unit (1 PCRU) per 10 μL and the RT-PCR titer was back-calculated to represent the initial sample concentration (PCRU/mL). This calculation of RT-PCR titer (PCRU/mL) was repeated at time zero and at each of the sampling points. The RT-PCR titer at each sampling point was divided by the RT-PCR titer at time zero and the ratio was $\log_{10}$ transformed. The $\log_{10}$ reduction in amplicon signal was plotted as a function of UV dose.

5.0 RESULTS

5.1 Overview

UV disinfection of Norwalk virus and two model enteric viruses, MS2 and poliovirus, was studied at UV doses up to 550 mW-sec/cm^2. The inactivation of MS2 and poliovirus was determined initially both by cell infectivity assay as well as RT-PCR assay of short genomic targets (<300 bp). For NV, which currently lacks a host cell line for plaque assay and which is without an animal model, RT-PCR was the only available alternative to measure reduction by UV. Comparison of PV and MS2 viral reduction as measured by RT-PCR amplification of short genomic targets with cell infectivity assay measurement of virus inactivation confirms the need to improve RT-PCR amplification techniques to better estimate UV disinfection. This is because the RT-PCR titer reductions were much less than the infectivity titer reductions at all UV doses. Therefore, for non-culturable viruses like NV, typical short product RT-PCR underestimates the loss of virus infectivity produced by UV. In subsequent experiments, attempts were made to overcome this problem by using immunocapture RT-PCR and long target RT-PCR.

5.2 Short Target RT-PCR Detection of Virus Reduction by UV Radiation

Direct, heat-release RT-PCR (D/RT-PCR) is a sensitive technique capable of detecting low levels of viral nucleic acid. It has a theoretical detection limit of 1 viral genomic unit per sample volume analyzed. The D/RT-PCR amplification of short viral

genomic targets for all three viruses was replicated in four UV radiation experiments (Table 5.1).

Amplification of a short PV genomic target (197 bp) showed only a 0-1 $\log_{10}$ reduction in RT-PCR amplicon signal at all doses, even after a UV dose of 550 mW-sec/cm^2. Similarly, the amplification of a short (232 bp) MS2 genomic target by D/RT-PCR detected a 0-1 $\log_{10}$ reduction in amplicon signal after a UV dose of up to 550 mW-sec/cm^2.

NV short target amplification, using the pol primer pair (260 bp) showed a greater loss of amplicon signal by UV radiation, relative to PV and MS2. After a UV dose of 30 mW-sec/cm^2, NV had a mean 0.5 $\log_{10}$ unit reduction of amplicon signal compared to no amplicon signal loss for PV and MS2. After a UV dose of 550 mW-sec/cm^2, RT-PCR amplification of the NV pol primer region showed a mean 1.5 $\log_{10}$ greater reduction in amplicon signal than was observed for PV or MS2 (2.5 $\log_{10}$ versus mean 1.0 $\log_{10}$ reduction, respectively).

5.3 Viral Reduction from UV Radiation Detected by Short Target RT-PCR Amplification and Virus Inactivation by Cell Infectivity Assay

There was little agreement between UV reduction of PV and MS2 as detected by loss of D/RT-PCR amplicon signal and the loss of viral infectivity titer. As seen in Table 5.1, poliovirus, was inactivated by 3.6 $\log_{10}$ after a UV dose of 30 mW-sec/cm^2. The detection limit of PV inactivation by UV radiation, a $\geq$5.5 $\log_{10}$ loss in virus infectivity titer, was reached after a UV dose of 90 mW-sec/cm^2. Based on cell infectivity data, MS2 was more resistant to UV inactivation than PV. For MS2 infectivity titer reductions by UV were 1.1 $\log_{10}$ at a UV dose of 30 mW-sec/cm^2, 4.8 $\log_{10}$ at a dose of 150 mW-

sec/cm^2 and the detection limit of ≥6.6 $\log_{10}$ at a dose of 350 mW-sec/cm^2.

The substantial reductions in PV and MS2 infectivity at progressively higher UV doses contrasts with the 0-1 $\log_{10}$ reductions in RT-PCR amplicon signal seen both for PV and MS2 at all UV doses. It appears that RT-PCR amplifies RNA associated not only with intact, infectious viruses but also with RNA from damaged, noninfectious virions and perhaps naked RNA. Therefore, efforts were made to investigate alternative RT-PCR methods to improve the relationship between the data for loss of infectivity and loss of RT-PCR amplicon signal from UV radiation.

5.4 UV Disinfection by Infectivity Assay and UV Virus Reduction as Detected by Alternative RT-PCR Amplification Methods

5.4a UV reduction of virus detected by infectivity and RT-PCR of multiple short genomic targets:

Amplification of multiple short viral genomic targets was performed in an attempt to improve RT-PCR detection of UV virus reduction. The amplification of multiple short targets increased the total length of amplified viral RNA, with the intent to improve the probability of detecting UV damage to the genome. Two MS2 short genomic targets (232bp, 233bp) and three NV short targets (224 bp, 470 bp, 224 bp) were amplified in two replicate experiments on UV radiation (Table 5.2). Amplification of multiple NV short targets with D/RT-PCR did not greatly improve detection of virus reduction by UV radiation, as is seen in Table 5.2. D/RT-PCR detected a 0-1 (mean 0.67) $\log_{10}$ reduction in amplicon signal at a UV dose of 90 mW-sec/cm^2, a 1 $\log_{10}$ loss at 50 mW-sec/cm^2 and a 2 $\log_{10}$ loss at 550 mW-sec/cm^2 for all three NV amplicons. These

results are quite similar to those of a single NV short genomic target (pol) in Table 5.1, with $\log_{10}$ RT-PCR titer reductions of 0.5, 1 and 2.5 at the same UV dose of 90, 150 and 550 mW-sec/cm^2, respectively. The two short genomic MS2 amplicons detected 0-1 $\log_{10}$ loss of signal at all UV doses (Table 5.4). These reductions of MS2 titer are nearly the same as the RT-PCR titer reductions obtained by amplification of a single short target as shown in Table 5.1. For MS2, corresponding reductions of infectivity by UV radiation also were similar to those in Table 5.1 and much greater than RT-PCR titer reductions.

5.4b UV reduction of virus detected by infectivity and AC/RT-PCR of multiple short genomic targets:

Antibody capture RT-PCR (AC/RT-PCR) was utilized in an effort to improve detection of intact, infectious viruses by RT-PCR amplification of only RNA released from immunomagnetically captured virions. AC/RT-PCR data was replicated in two experiments for the three NV amplicons, two MS2 amplicons and one PV short target amplicon (Table 5.5).

AC/RT-PCR amplification of PV RNA with the pan-Entero primer pair (197 bp) did not alter RT-PCR detection of virus reduction compared to D/RT-PCR, even after a UV dose of 550 mW-sec/cm^2. With both D/RT-PCR and AC/RT-PCR amplification, 0-1 $\log_{10}$ loss of amplicon signal was observed for PV at all UV doses (Table 5.5). Similarly, MS2 reduction, as detected by Rep 1 and Rep 2 amplicon loss by RT-PCR end-point titration, did not improve with AC/RT-PCR relative to D/RT-PCR (Table 5.4). In fact, at a dose of 150 mW-sec/cm^2, AC/RT-PCR showed less reduction in the MS2 amplicon signals than D/RT-PCR (0 $\log_{10}$ versus 1 $\log_{10}$).

Reduction of Norwalk virus by UV radiation, as based on reduction of all three AC/RT-PCR amplicon signal endpoints, is shown in Table 5.2. After a UV dose of 150 mW-sec/cm^2 and higher, AC/RT-PCR amplification showed less reduction in NV amplicon signal than with D/RT-PCR amplification of the same NV pol amplicon. AC/RT-PCR detected a 0-1 $\log_{10}$ NV reduction in signal at all UV doses for all three NV short targets including a dose of 550 mW-sec/cm^2. For a UV dose of 550 mW-sec/cm^2, the AC/RT-PCR reductions in amplicon signals (0-1 $\log_{10}$) are 1-2 $\log_{10}$ less than the amplicon signal reductions detected by D/RT-PCR (2 $\log_{10}$).

5.4c UV reduction of virus detected by infectivity and RT-PCR of long genomic targets:

In order to increase the likelihood of detecting UV damage to the RNA viral template, the length of RT-PCR genomic targets was increased. The probability of detecting a loss of amplicon signal produced by UV damage to RNA template was thought to increase with a longer target. Two replicate experiments were performed with the amplification of two NV long targets, one PV long target and one MS2 long target.

As seen in Table 5.3, the RT-PCR amplification of a longer poliovirus target (970 bp) showed a greater reduction in amplicon signal than the amplification of a short PV genomic target. After a UV dose of 150 mW-sec/cm^2, the longer PV amplicon signal endpoint was reduced by 2 $\log_{10}$ while the short target amplicon signal was reduced only by 1 $\log_{10}$. After a UV dose of 550 mW-sec/cm^2, the longer PV amplicon signal was reduced by $\geq$3 $\log_{10}$ units in contrast to the 1 $\log_{10}$ reduction seen by short target RT-PCR end-point titration.

RT-PCR amplification of a longer MS2 amplicon (500bp) did not improve detection of virus reduction by UV radiation. As seen in Table 5.3, reduction in amplicon signal was essentially equivalent for RT-PCR amplification of long and short MS2 targets. At all doses, there was a 0-1 $\log_{10}$ loss in amplicon signal for both short and long targets. Therefore, the amplification of the long MS2 target by RT-PCR did not improve the relationship between short RT-PCR amplicon reduction and MS2 inactivation based on infectivity assay.

However, D/RT-PCR amplification of longer targets detected a greater reduction of NV than did the amplification short targets, especially at lower UV doses. As shown in Table 5.3, the $\log_{10}$ reduction of long (1347 bp) target D/RT-PCR signal was a 2 $\log_{10}$ after a dose of 30 mW-sec/cm^2 and the detection limit ($\geq$3 $\log_{10}$) was reached after a UV dose of 150 mW-sec/cm^2. For the second long NV amplicon (921 bp), the D/RT-PCR titer reductions were 1 $\log_{10}$ after a UV dose of 30 mW-sec/cm^2 and the detection limit of $\geq$2 $\log_{10}$ after a UV dose of 150 mW-sec/cm^2.

5.4d Comparison of UV reduction by alternative RT-PCR methods:

The detection of virus reduction by alternative RT-PCR techniques is summarized in Figure 5.1. Viral reduction as measured by loss of short D/RT-PCR amplicon signal did not correlate with PV1 and MS2 inactivation as determined by infectivity assays. By infectivity assay, a UV dose of 30 mW-sec/cm^2 resulted in measurable inactivation of PV and MS2 (3.6 and 1.1 $\log_{10}$, respectively), with the detection limit of virus inactivation ($\geq$6.6 $\log_{10}$ reduction for MS2 and $\geq$5.5 $\log_{10}$ reduction for PV) reached after a UV dose of 350 mW-sec/cm. For all three viruses, short target RT-PCR detected only a 1 $\log_{10}$

reduction in virus signal after a UV dose of 150 mW-sec/cm^2, in contrast to the 4.8-≥5.5 $\log_{10}$ reduction in infectivity titer for PV1 and MS2, respectively. D/RT-PCR amplification of short targets showed a greater reduction in amplicon signal for NV than for PV and MS2. This suggests that NV may be more sensitive to UV than these other enteric viruses.

Reduction of the PV long D/RT-PCR amplicon better estimated UV inactivation of PV than did D/RT-PCR amplification of the short target. At UV doses of 150 mW-sec/cm^2 and higher, amplification of the long PV amplicon improved the detection of virus reduction relative to short target amplification by 1-2 $\log_{10}$. For NV, amplification of long targets also showed a greater titer reduction than short targets at all UV doses tested. For MS2, long product and short product RT-PCR reductions of only 0-1 $\log_{10}$ were observed at all UV doses (30 to 550 mW-sec/cm^2). Indeed, RT-PCR amplification of the long product gave MS2 titer reductions which were either less than or no greater than those for the short product.

5.5 UV Disinfection of NV, Poliovirus and MS2 and Their Detection by Alternative RT-PCR Amplification Methods and Infectivity

Figure 5.2 and Figure 5.3 summarize UV disinfection of poliovirus and MS2 respectively, based on infectivity and RT-PCR assays. Figure 5.4 summarizes UV reduction of NV by RT-PCR alternative amplification techniques. As is seen in Figure 5.2, UV inactivation of poliovirus by cell infectivity assay, does not correlate with reduction of D/RT-PCR short amplicon signal. Antibody capture of the PV virion prior to RT-PCR amplification of the short PV genomic target did not improve the RT-PCR

estimation of PV inactivation by UV radiation. However, RT-PCR amplification of a long PV target improved the detection of PV reduction by UV relative to cell infectivity assay.

Improved detection of virus reduction by UV radiation using a longer genomic target for RT-PCR amplification was not observed for MS2. As shown in Figure 5.3, UV reduction of the long MS2 amplicon does not differ from the end-point titer reduction either by direct or AC/RT-PCR amplification of a short MS2 genomic target.

Figure 5.3 illustrates UV reduction of NV as detected by loss of RT-PCR amplicon signal. Antibody capture of the NV virion prior to amplification of short NV targets did not improve the detection of NV reduction by UV irradiation. However, amplification of long NV amplicon 1 detected the greatest reduction of NV with the detection limit reached at 150 mW-sec/cm^2. At UV doses less than 350 mW-sec/cm^2, reduction of NV long amplicon 2 and long amplicon 1 were 1-2 $\log_{10}$ greater than the reductions of NV short amplicon signal. Overall, UV radiation causes somewhat greater loss of NV titer by RT-PCR amplification of long genomic targets than short genomic targets.

Figure 5.1: UV Reduction (-Log_{10}) of NV, PV1, and MS2 Based on Infectivity Assay and RT-PCR Amplification Methods

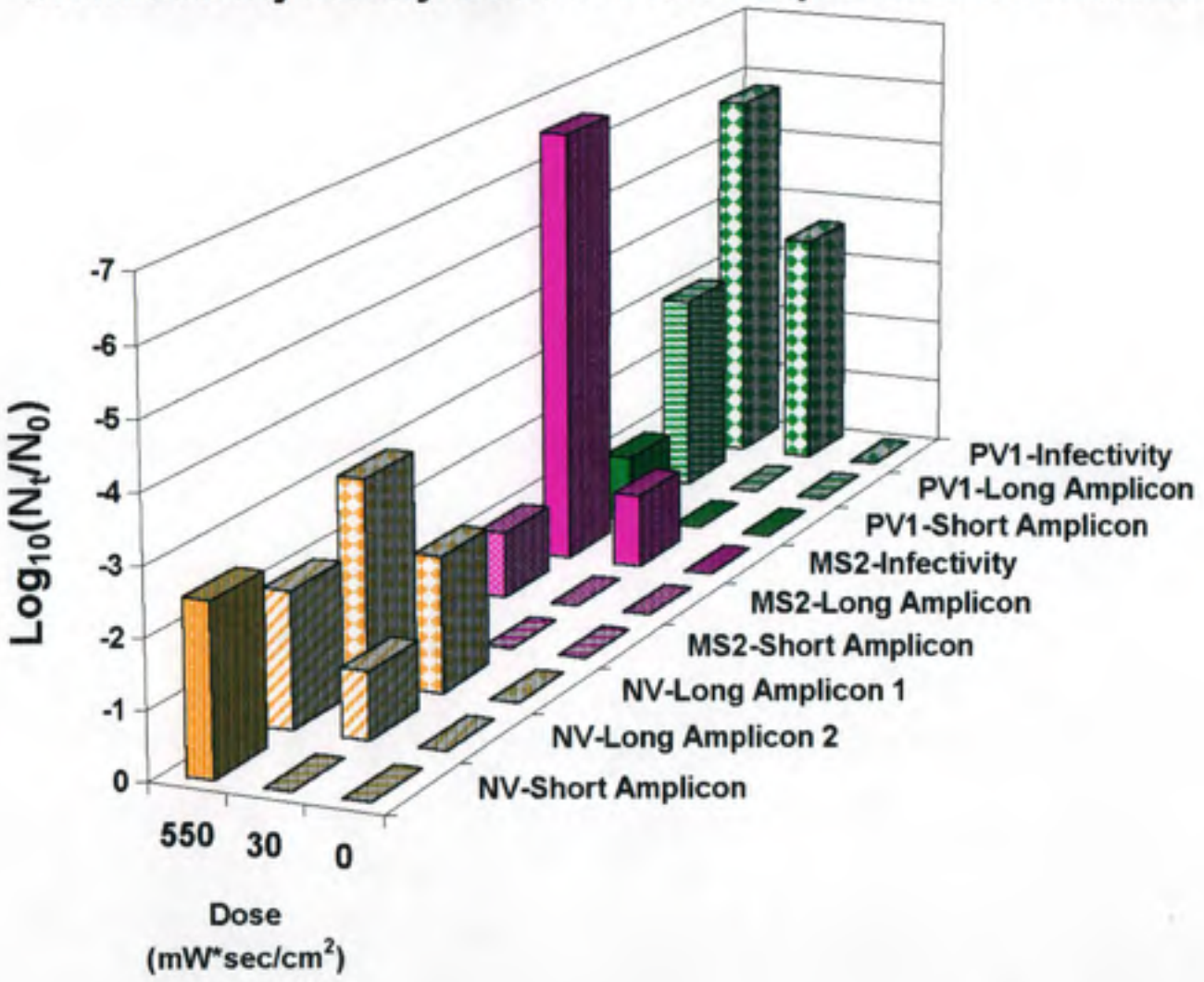

Figure 5.2: UV Reduction (-Log_{10}) of Poliovirus Based on Infectivity and Alternative RT-PCR Assays

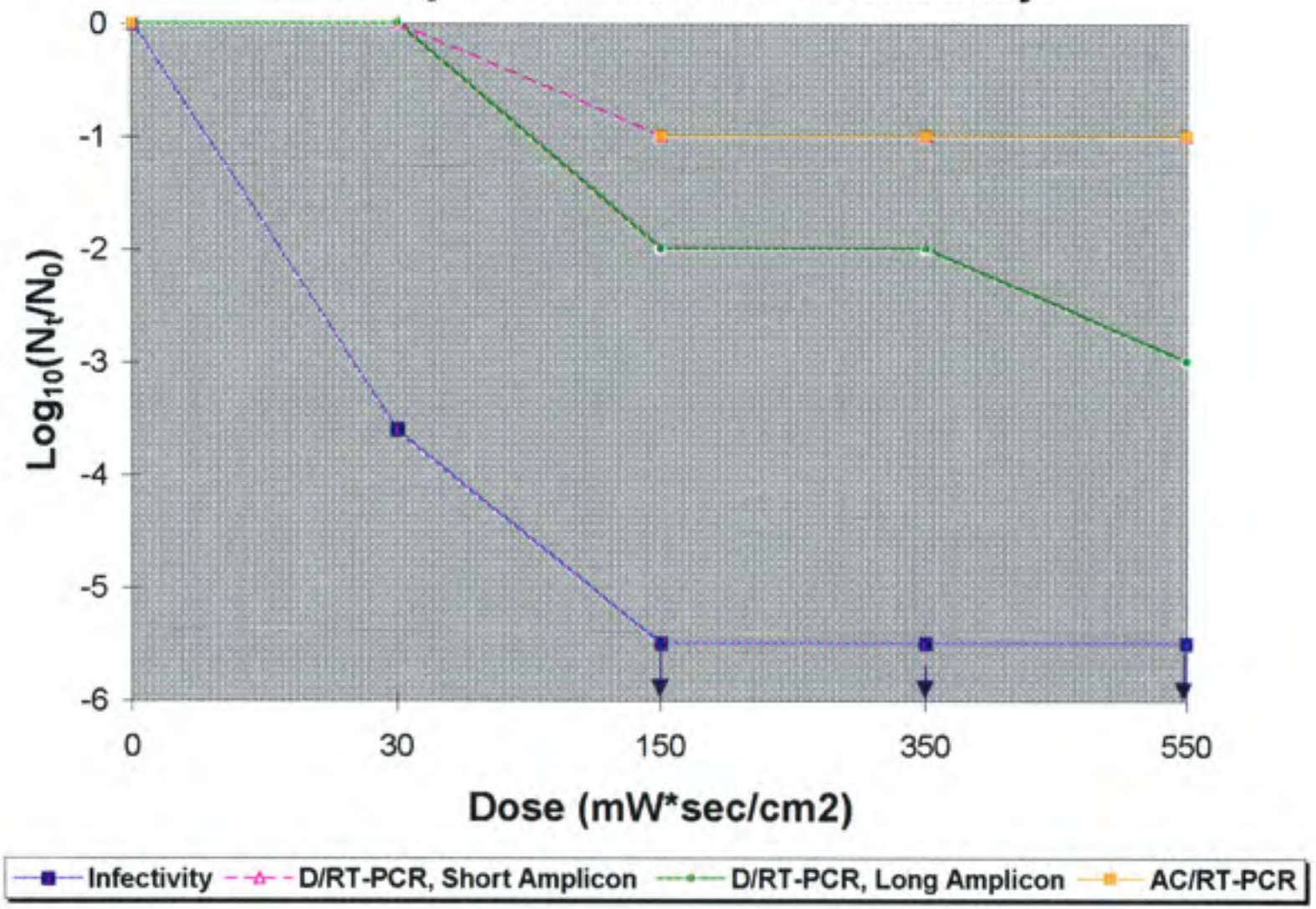

Figure 5.3: UV Reduction (-Log_{10}) of MS2 Based on Infectivity and Alternative RT-PCR Assays

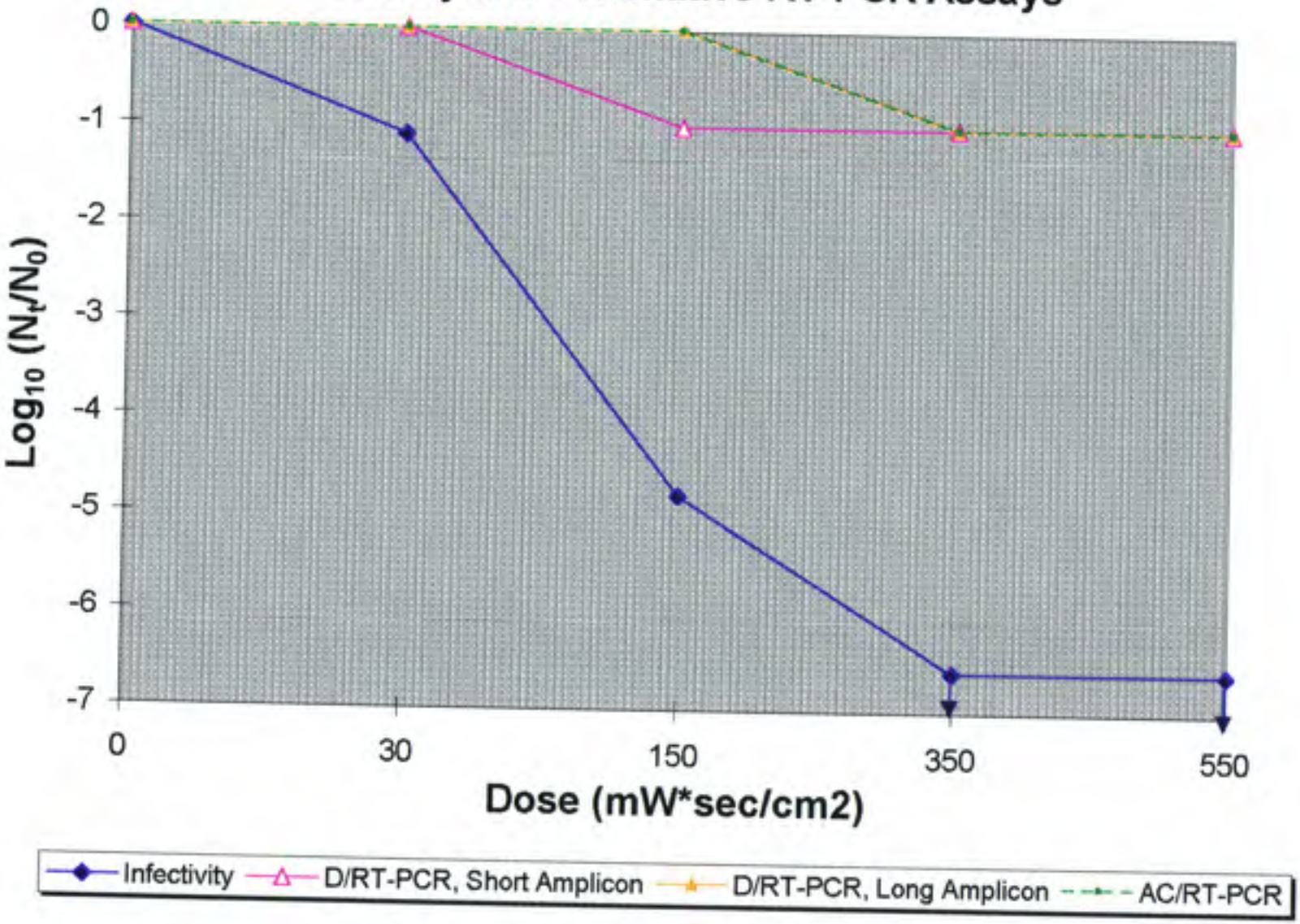

Figure 5.4: UV Reduction (-Log_{10}) of Norwalk Virus Based on Alternative RT-PCR Assays

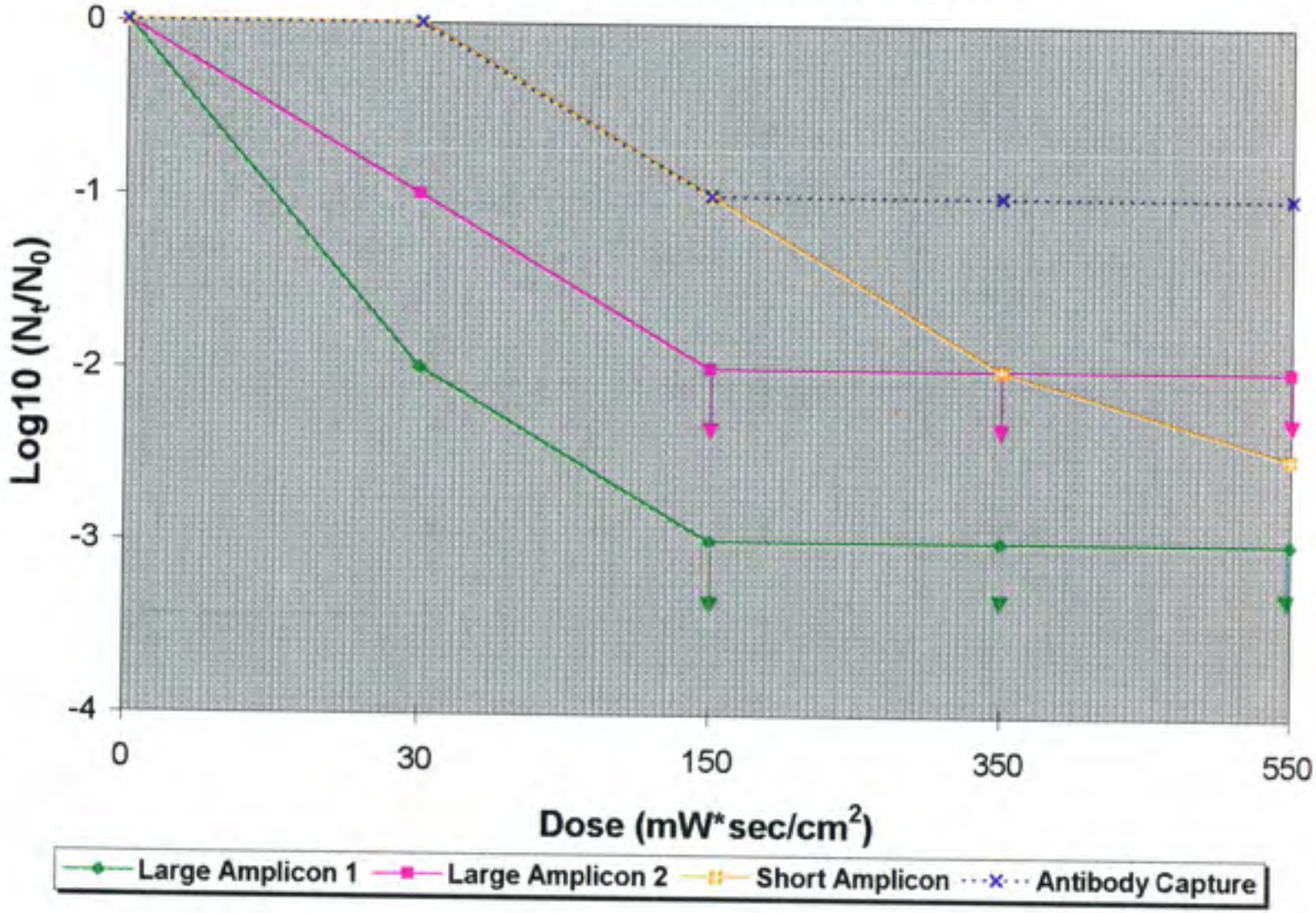

Table 5.1: Reduction (-Log_{10}) of Norwalk Virus, Poliovirus and MS2 by UV Radiation by Infectivity Assay and Short Target RT-PCR[a]

	Norwalk virus		Poliovirus		MS-2	
Dose mW*sec/cm^2	RT-PCR	infectivity	RT-PCR	infectivity	RT-PCR	infectivity
0	0	N/D	0	0	0	0
30	0	N/D	0	3.6	0	1.1
90	0.5	N/D	0	>5.5[b]	0	3.0
150	1	N/D	1	>5.5[b]	1	4.8
350	2	N/D	1	>5.5[b]	1	>6.6[b]
550	2.5	N/D	1	>5.5[b]	1	>6.6[b]

[a]Amplicon size (bp) for primer targets: NV pol = 260, PV pan Entero = 196, MS-2 Rep 1 = 234; mean results of 4 trials

[b]Includes detection limit for one or more trials

N/D = No Data

Table 5.2: Reduction (-Log_{10}) of Norwalk Virus by UV Radiation at Multiple Targets by Direct and Antibody Capture RT-PCR[a]

	Direct (heat release) PCR				Antibody Capture PCR			
(Dose) mW-sec/cm^2	Pol	IP	35/36	Avg	Pol	IP	35/36	Avg
0	0	0	0	0	0	0	0	0
90	1	0	1	0.67	0	0	1	0.33
150	1	1	1	1	1	0	1	0.67
250	1	1	1	1	N/D	N/D	N/D	N/D
350	2	1	1	1.3	N/D	N/D	N/D	N/D
550	2	2	2	2	1	0	1	0.67

[a]Amplicon size (bp) for primer targets: NV pol =260, IP = 224, 35/36 = 470; results of duplicate experiments

N/D = No Data

Table 5.3: Reduction (-Log_{10}) of NV, PV and MS2 by Long Target and Short Target RT-PCR and Infectivity Assay

	Norwalk virus			Poliovirus			MS-2		
UV Dose	D/RT-PCR			D/RT-PCR		Infectivity[a]	D/RT-PCR		Infectivity[a]
mW*sec/cm2	Long 1[b]	Long 2[b]	Short[a]	Long 1[b]	Short[a]		Long[b]	Short[a]	
0	0	0	0	0	0	0	0	0	0.0
30	2	1	0	0	0	3.6	0	1	1.1
150	>3	>2	1	2	1	>5.5	0	1	4.8
350	>3	>2		2		>5.5	1		>6.6
550	>3	>2	2.5	>3	1	>5.5	1	1	>6.6

[a]Tabulated values are means from four replicate experiments

[b]Tabulated values are means from two replicate experiments

Target Sizes (bp): NV Long 1 = NV2A/B (1347 bp), NV Long 2 = NV C3/C5 (922), NV Short = pol (260), PV Long = pol C/D (970), PV Short = pan-entero (197), MS2 Long = BREP 3/5 (494), MS2 Short = Rep 1 (233)

Table 5.4: Reduction (-Log_{10}) of MS2 by D/RT-PCR and AC/RT-PCR Amplification of Short Targets[a]

	D/RT-PCR		AC/RT-PCR	
Dose mW*sec/cm^2	Rep 1	Rep 2	Rep 1	Rep 2
0	0	0	0	0
30	1	0	N/D	N/D
90	1	0	0	0
150	1	1	0	0
550	1	1	1	0

[a]Amplicon size (bp) for primer targets: MS2 Rep 1 = 234; MS2 Rep 2 = 233;
N/D = No Data

Table 5.5: Reduction (-Log_{10}) of NV, PV and MS2 by AC/RT-PCR

Dose mW*sec/cm^2	PV-panE	NV pol	NV IP	NV 35/36	MS2-Rep1	MS2-Rep2
0	0	0	0	0	0	0
90	0	0	0	1	0	0
150	1	1	0	1	1	0
550	1	1	0	1	1	1

[a]Amplicon size (bp) for primer targets: MS2 Rep 1 = 234; MS2 Rep 2 = 233;
NV pol = 260, IP = 224, 35/36 = 470, panEntero = 197

N/D = No Data

6.0 DISCUSSION

Present and forthcoming drinking water regulations by the US EPA place greater responsibility on water treatment utilities to meet disinfectant and disinfection by-product MCLs while maintaining the microbial quality of finished water. Ultraviolet radiation may become an increasingly attractive alternative to chlorine for drinking water disinfection. This may be especially applicable to for groundwater sources of drinking water, where the greatest microbial risk is from enteric viruses. However, if the efficacy of UV disinfection of protozoan cysts can be increased with UV technologies such as medium pressure, high intensity lamps and pulsed, broad wavelength spectrum irradiation, UV radiation may also gain greater acceptance for disinfecting surface water systems.

Improved methods are needed to better detect and monitor microbial pathogens in drinking water supplies. Limitations of conventional infectivity assays, particularly for important, non-culturable viral pathogens such as Norwalk virus, motivates current efforts to improve and adapt molecular techniques such as RT-PCR for detection of low levels of infectious viruses in drinking water and other environmental samples. At present, RT-PCR amplification is severely limited by its inability to reliably estimate concentrations of infectious viruses. As shown by Maier (1995) for UV irradiated poliovirus and as well as for bacterial pathogens by Josephson (1993), current PCR techniques will amplify and detect nucleic acid from infectious as well as noninfectious pathogens.

Study of UV disinfection of NV is limited by its lack of an infectivity assay and the difficulty of interpreting RT-PCR results in terms of infectious viral units. Consequently,

a key task of this study was to improve the relationship between UV inactivation of model viruses (PV and MS2), as determined by cell infectivity assay, and RT-PCR amplicon signal reduction.

Infectivity assay confirmed the unusual UV resistance of MS2 as noted in previous reports. Wiedenmann (1993) and Battigeli (1993) both noted that the UV dose necessary for a 4 $\log_{10}$ inactivation of MS2 was 3 times greater than for HAV. Meng (1996) reported that MS2 was less sensitive to UV radiation than PV1. This study observed a 3.6 $\log_{10}$ reduction in PV and a 1.1 $\log_{10}$ reduction in MS2 at 30 mW-sec/cm^2. The extent of poliovirus reduction seen in this study is consistent with the previous work by Maier (1996) and Meng (1996) who reported 4 $\log_{10}$ inactivation of PV1 infectivity by UV doses of 30 and 21.7 mW-sec/cm^2, respectively. Chang (1985) detected a 3 $\log_{10}$ PV inactivation at a dose of 30 mW-sec/cm^2. Typically, viruses with single-stranded nucleic acid are more sensitive to UV damage and more rapidly inactivated than viruses with double-stranded nucleic acid. However, MS2, a single-stranded RNA virus showed UV resistance equivalent to double stranded DNA viruses (Meng, 1996). This unusual resistance to UV radiation may be due in part to its small genome size (~4000 bp), very compact RNA structure and high degree of hydrogen bonding, as noted by Strauss, (1963).

As expected, the reduction of the short RT-PCR amplicons did not reflect the loss of infectivity for UV irradiated PV and MS2. For both viruses, RT-PCR end-point titration detected a 0-1 $\log_{10}$ loss of short amplicons at all doses. By plaque assay, after a UV dose of 550 mW-sec/cm^2 there was a 5.5 $\log_{10}$ loss of PV infectivity titer and 6.6 $\log_{10}$ loss of MS2 infectivity titer.

Estimation of UV inactivation of MS2 infectivity did not improve with D/RT-PCR amplification of multiple short genomic targets. This inability of RT-PCR to detect MS2 inactivation may be a consequence of the stable secondary structure of its RNA genome. This secondary structure may protect the genomic RNA from the formation of UV generated photoproducts in the regions amplified.

The amplification of multiple short NV genomic RNA targets by D/RT-PCR did not increase the detection of virus reduction by UV irradiation.

In order to improve the detection of UV reduction of virus infectivity, antibody capture of intact virions prior to RT-PCR amplification was investigated. As observed by Limsawat *et al.* (1997), liberated viral RNA can be stable in autoclaved, sterile water and filtered activated sludge for 18 and 5 days respectively. This free viral RNA is detectable by RT-PCR. Antibody capture of viruses prior to RT-PCR recovered only viruses with intact capsid epitopes, capable of an antibody-antigen reaction. This technique should exclude the capture and amplification of free RNA not associated with virions and consequently not presenting a public health risk.

The antibody capture of NV virions with polyclonal antibodies bound to paramagnetic beads prior to RNA heat-release and RT-PCR amplification gave NV reductions by UV radiation that were similar to those obtained by D/RT-PCR at multiple short viral genomic targets. AC/RT-PCR amplification of UV irradiated PV was equivalent to D/RT-PCR amplification using the same primer pair. Detection of MS2 reduction by UV radiation using multiple short D/RT-PCR targets also did not improve with AC/RT-PCR amplification. The ineffectiveness of AC/RT-PCR for detection of viral inactivation by UV radiation may result from a lack of sensitivity to UV damaged virions.

The nucleic acid is likely more quickly damaged than the viral capsid, reducing infectivity titer at doses well below the UV doses required of effect reduction in RT-PCR amplicons.

The recent development of monoclonal antibodies against Norwalk virus may provide a more specific solid-phase antibody capture technique of a unique NV epitope. The capture of a NV epitope, specific to an attachment site affected by UV radiation damage, could enable an immunomagnetic capture technique capable of distinguishing infectious from non-infectious Norwalk virions. This specificity of monoclonal antibodies for immunomagnetic capture could provide RT-PCR detection of UV irradiated enteric viruses that better estimated UV irradiation of the viruses.

The amplification of long viral RNA targets by direct (heat-release) RT-PCR improved the estimation of PV inactivation. At a UV dose of 550 mW-sec/cm^2, there was a 2 $\log_{10}$ greater reduction in long amplicon than was seen with the short target amplicon. Surprisingly, the RT-PCR amplification of longer RNA targets by RT-PCR did not improve the detection of UV reduction of MS2. Long target RT-PCR amplification detected a greater reduction of NV at lower UV doses of 30 mW-sec/cm^2, and 150 mW-sec/cm^2. However, the difference in amplicon reduction between the short and long NV genomic targets diminished at a UV dose of 550 mW-sec/cm^2.

The RT-PCR amplification of large viral genomic targets shows the potential to improve the detection of UV inactivation of viruses. Increasing the length of viral genome amplified by RT-PCR increases the likelihood of detecting a region impacted by UV damage to the nucleic acid. In this study, primer pairs amplified 6-13% of the MS2 RNA genome, 3-18% of the NV RNA genome and 3-15% of the PV RNA genome.

Increasing the amplicon length may increase the probability of detecting UV damage by loss of PCR signal and consequently may better estimate viral inactivation by UV radiation. Additional research is needed to determine whether primer pairs to amplify longer targets can be designed to detect low levels of virus and to investigate whether even longer targets for RT-PCR amplification of viral nucleic acids are even better correlated loss of virus infectivity from UV radiation.

7.0 CONCLUSIONS

1. RT-PCR results from UV irradiated viruses cannot be interpreted as an estimation of microbial risk. Target nucleic acid from noninfectious viral units is detected in addition to those from infectious virions.
2. UV radiation efficiently inactivated poliovirus under laboratory conditions, with a 3.7 $\log_{10}$ reduction detected by cell infectivity assay after a UV dose of 30 mW-sec/cm^2. MS2 showed a higher resistance to UV inactivation with a 1.1 $\log_{10}$ reduction detected at the same dose. Norwalk virus, currently without a cell culture infectivity assay, requires a molecular technique such as RT-PCR for sensitive detection. At a UV dose of 30 mW-sec/cm^2, there was no reduction of RT-PCR titer of NV when amplifying a short genomic target. The same was true for PV1 and MS2.
3. RT-PCR amplification of multiple short targets for PV, NV and MS2 did not significantly improve the detection of viral inactivation by UV radiation, with 0-1 $\log_{10}$ reduction detected for all viruses at all doses.
4. Antibody capture of virions prior to RNA release either reduced or did not improve the detection of PV and MS2 inactivation by UV radiation and reduced the detection of NV titer reduction.
5. MS2 coliphage did not show much reduction in titer by either antibody capture RT-PCR or direct RT-PCR of short and long genomic targets.
6. Additional research to evaluate the impact of increased target viral RNA size for RT-PCR on estimation of viral inactivation by UV radiation is needed.
7. The application of monoclonal antibodies rather than polyclonal antisera in the immunomagnetic capture of intact virions prior to RT-PCR amplification may improve specificity and thereby provide better detection of UV inactivation.

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