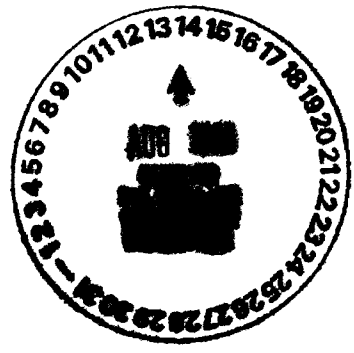

DETECTION OF ENTERIC VIRUSES IN TREATED WASTEWATER SLUDGE USING CELL CULTURE AND MOLECULAR METHODS

by

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ABSTRACT

Two continuous cell lines, BGM and CaCo-2 were compared for the detection of viruses in mesophilic treated sludge (MTS) and secondary disinfected wastewater effluent (WWE) samples. Samples were inoculated in both cell lines and examined microscopically for cytopathogenic effect (CPE) for 14 days. Enumeration by the most probable number (MPN) method and statistical analysis revealed significantly greater MPN values for CaCo-2 than in BGM cells for WWE. Statistical analysis of MTS and WWE samples showed that CaCo-2 cells were more sensitive than BGM ($p=0.0287$). This suggests that CaCo-2 cells are more sensitive for the detection of enteroviruses in environmental samples. M-PCR was developed to detect and differentiate human adenovirus (Ad) from enteric adenovirus (Ead) from seeded environmental samples. Two sets of primers hexAA1885/1913 and K402/403 (308 bp and 152 bp amplicons respectively) were chosen for combination in M-PCR. The optimum $MgCl_2$ concentration was 1.25 mM with primer concentration of 100 pmol for hexAA1885/1913 and 50 pmol for K402/403 primers. Optimum primer annealing temperature was 60° C. Sensitivity of M-PCR was 10^2 $TCID_{50}$ for Ead and Ad mixed and 10^0 $TCID_{50}$ for individual viruses per reaction. M-PCR has potential in the rapid and specific identification of these types of viruses in environmental samples.

CHAPTER 1 INTRODUCTION

The Federal Water Pollution Control Act of 1972 emphasized the need to employ environmentally sound sludge management practices and mandated a minimum of secondary treatment of municipal sludge. This resulted in huge increases in the amount of sewage sludge generated. Focused attention on the disposal needs and potential benefits of the application of sewage sludge to agricultural soils became a realistic method. In 1989 the United States Environmental Protection Agency (USEPA) announced proposed standards for the reduction of pathogens during the sludge treatment process (USEPA, 1989). Municipalities became pressured to upgrade treatment to meet sludge reduction requirements. At that time the number of treatment facilities equipped with laboratories and trained personal to monitor for pathogens was inadequate. In addition to the proposed standards, the USEPA would also ban the disposal of sewage sludge into any body of water, fresh or marine, leaving land disposal or incineration as the only options. This further increased the burden on landfill space outside of metropolitan areas.

Pima County, Arizona is currently investigating the benefits of the application of treated sewage sludge on agricultural fields. However treated sludge may still contain enteric virus concentrations of up to 1000 / L. Applied as a liquid with approximately 1-2% solids, sludge contributes water and fertilizer to the soil. Problems from the application of sludge to soils is the potential impact on groundwater from infiltration or to

surface waters from agricultural run off. This scenario could result in the contamination of ground or surface waters with enteric viruses.

The assay of enteric viruses in sludge involves the use of continuous mammalian cell cultures for detection. This method of virus detection is expensive and requires a minimum of two weeks to completion. Currently there is only one cell culture line routinely used for the detection of enteric viruses, even though a large variety of cell lines are available. Several of these cell lines are permissive to infection by enteric viruses but have not been evaluated for sensitivity using environmental samples.

The Polymerase Chain Reaction (PCR) has been utilized as a presumptive test for the detection of viruses. PCR involves the detection and amplification of intact nucleic acid sequences specific for enteric viruses in sludge samples. Recently, Multiplex Polymerase Chain Reaction (M-PCR) has been developed for the detection of more than one specific nucleic acid sequence in one reaction (Mahoney and Chernesky, 1995). The advantages to this method are the reduced costs compared to cell culture as well as the ability to detect two or more types of virus in one reaction. Furthermore, the results are available within 24 hours. This method however cannot determine infectivity of the virus.

This work compared the survival of enteric viruses (including enteric adenoviruses) in treated sludge by using two different cell lines. In conjunction, a method to detect enteric adenoviruses using M-PCR in sludge was also developed. The combination of cell culture and nucleic acid detection systems provide a comprehensive analysis of the virus content in treated sewage sludge.

CHAPTER 2 LITERATURE REVIEW

The Origin of Sewage Sludge (Wastewater Process)

Sewage sludge is a complex mixture of biosolids derived from precipitation processes during the various phases of sewage treatment. Raw sewage entering the wastewater treatment facility is first passed thorough screen and a grit chamber to remove lager debris such as sand, bone chips, wood and glass. These materials are either caught in the screen or settle in the grit chamber and are directly disposed of in a landfill (Bitton, 1994). Primary treatment of sewage is a physical process where suspended solids are allowed to settle and are accumulated in a primary clarifier. These solids are termed primary sludge (Hurst, 1988). Primary effluent is further treated by biological processes to reduce biochemical oxygen demand, potential pathogens and odor. Several methods can accomplish this such as trickling filter, activated sludge, or rotating biological contractors. During treatment, organic matter is converted to CO_2, H_2O and microbial biomass. The excess biomass from these processes is termed secondary sludge and contains mostly organic matter. Removal of secondary sludge is accomplished mostly by settling. Where advanced wastewater treatment is performed, alum [$Al_2(SO_4)_3 \bullet 18H_2O$], ferric chloride ($FeCl_3$) or lime [$Ca(OH)_2$] is added to secondary effluent. Solids produced from the flocculation of chemical constituents such as phosphates are termed tertiary sludge. Sewage sludge is then a mixture of primary, secondary and tertiary sludges depending on the level of treatment performed (Straub, 1993).

Sewage sludge must be treated to stabilize organic decomposition, gas production and to reduce the concentration of pathogens. Anaerobic digestion consists of a series of microbiological processes that covert organic compounds to methane. Anaerobic digestion produces 3-20 times less sludge compared to aerobic digestion but it is a slower process (Bitton, 1994). Anaerobic digestion is operated semi-continuously in large enclosed tanks called sludge digestors, into which untreated material is introduced and treated material is removed at intervals. Retention time in the tank may range from two weeks to a month. The USEPA defines anaerobic digestion as a process conducted in the absence of air at residence times ranging from 60 days at 20° C to 15 days at 35° to 55° C with reduction of volatile solids of at least 38 % (USEPA, 1989). In contrast, aerobic digestion stabilizes sludge by the passage of air through the sludge in a reactor. Aeration in this manner occurs for two to three weeks.

Pathogens of Concern

Sewage may contain a wide variety of microorganisms that are pathogenic to humans. The pathogens of concern include bacteria, viruses, protozoa, helminths and fungi, all of which are expected to be present in raw, primary, and secondary sludges. Table 2.1 lists the viral pathogens of concern. The list of pathogens is not constant as advances in analytical techniques and changes in society either recognize new pathogens or the significance of identified pathogens changes. Microorganisms evolve allowing for adaptation to changes in their environment. In addition, many bacterial pathogens are viable but non-culturable using current techniques (Rozak and Colwell, 1987) and actual

TABLE 2.1: Some Human Enteric Viruses Found in Feces

Virus Group	Number of serotypes	Associated illness
Adenoviruses	49	Pharyngitis, conjunctivitis respiratory illness, vomiting, diarrhea, gastroenteritis
Astrovirus	5	Gastroenteritis
Calcivirus	2	Vomiting, diarrhea
Coronavirus	1	Vomiting, diarrhea
Enterovirus:		
Polio	3	Paralysis, meningitis, fever
Coxsackie A	23	Herpangina, respiratory illness, meningitis, fever
Coxsackie B	6	Myocarditis, congential heart anomalies, rash, fever, meningitis, respiratory illness, pleurodynia
Echovirus	32	Meningitis, encephalitis, respiratory disease, rash, diarrhea, fever
Enterovirus (68-71)	4	Meningitis, encephalitis, respiratory illness, acute hemorrhagic conjunctivitis, fever
Hepatitis A virus	1	Hepatitis
Hepatitis E virus	1	Hepatitis
Norwalk agent	1	Epidemic vomiting and diarrhea
Reovirus	3	Respiratory disease
Rotavirus	4	Vomiting, diarrhea
"Small round viruses"	2	Vomiting, diarrhea

Modified from Bitton (1980), Hurst (1988), Jehl-Pietri (1992), and Schwartzbrod et al. (1990)

concentrations in sludge are presumably underestimated. Thus the assessment of the microbial risks associated with sludge disposal cannot be considered complete. As a greater understanding of microbial ecology is developed, researchers must be willing to reevaluate previous assumptions.

Viral Pathogens

Over 140 different viruses may be excreted in human feces and urine which find their way in sewage. A listing of viruses and the diseases they cause which may be present in sewage can be found in Table 2.1. Enteric viruses are those that can replicate in the gastrointestinal tract and shed in the feces. They are differentiated into several groups based on morphological, physical, biochemical, genetic, and antigenic characteristics. Infected individuals may excrete 10^{10} viruses per gram of feces for up to 50 days (Melnick and Gerba, 1980).

Sewage entering wastewater treatment contains significant numbers of viral pathogens. Enteric virus densities in the U.S. are estimated to be in excess of 7000 / L of raw sewage (Melnick et al., 1978) which are then concentrated in sludge (Ward and Ashley, 1977; Wellings et al., 1976). Treatment of sludge may not effectively reduce the number of infectious viruses. Soares (1990) found that viral reduction in anaerobically digested sludge ranged from less than 50 % to greater than 99.9 % imparting a high degree of variability in treatment efficiency. If treatment efficiency were maintained at 99 % reduction; the number of viruses in a typical treated sludge could still be in excess of

1000 / L. Wellings et al. (1976) reported concentrations of 24 plaque forming units (pfu) of virus per 250 g of sludge cake. Another study by Payment et al. (1986) found viruses in 94 % of sludge samples.

Most of the research on viruses in sewage focuses on those associated with gastroenteritis. Exceptions are certain enteroviruses with a wide variety of associated diseases and adenoviruses which may cause eye and upper respiratory disease as well as gastroenteritis.

The most frequently studied enteric viruses in sludge are the enteroviruses which include: poliovirus, coxsackie A and B virus, echoviruses and other recently classified enteroviruses. Most of the enterovirus infections, such as those caused by poliovirus, may be asymptomatic; however, during outbreaks of hepatitis, another waterborne virus, symptomatic infections may be as high as 95 % (Lender et al., 1985). Many studies have been conducted on the occurrence of enteroviruses in sludge as well as their removal by sewage treatment (Leong, 1983).

Rotavirus are now recognized as the leading cause of childhood gastroenteritis, sometimes resulting in dehydration and death in infants and adults (Gerba et al., 1985). Several waterborne outbreaks have been documented (Gerba et al., 1985; Williams and Akin, 1986) and the virus isolated from sewage sludge (Gerba, 1986).

Adenoviruses cause primarily eye and upper respiratory infections. These viruses have been identified as the cause of death in transplant and AIDS patients (Durepaire et al., 1995; Shields et al., 1985; Zahradnik et al., 1980). Several waterborne outbreaks have been associated with swimming pools and recreational lakes (MMWR, 1992;

Caldwell et al., 1974). Of increasing concern are enteric adenovirus (Ead) types 40 and 41 which have been shown to be the second leading cause of acute gastroenteritis in children (Herrmann et al., 1988; Uhnoo et al., 1984); however, no waterborne outbreaks of Ead 40 / 41 have been documented.

Norwalk virus has been demonstrated to be the cause of numerous waterborne outbreaks of epidemic gastroenteritis (Gerba and Bitton, 1984). Currently, there are no methods for isolation of this agent in cell culture so its occurrence and concentration in sewage sludge is unknown. Astroviruses, caliciviruses, coronaviruses and several other norwalk-like agents have been associated with human gastroenteritis, but little information exists on their occurrence in sewage sludge or wastewater.

The Adenoviruses

Adenoviruses are double stranded DNA icosahedral viruses approximately 70 nm in diameter (Fields, 1996). Currently 49 human adenoviruses have been identified (Fields, 1996). Adenoviruses can cause acute respiratory disease, pneumonia, epidemic conjunctivitis, and acute gastroenteritis (Uhnoo et al., 1983). Waterborne outbreaks have been documented for adenoviruses type 3 (Martone et al., 1980) and type 4 (D'Angelo et al., 1979). In the United States from 1991 to 1992 outbreaks of adenovirus type 3 caused conjunctivitis, pharyngitis, and fever affecting 595 individuals (Moore et al., 1993; McMillan et al., 1992).

Adenovirus appear to be more stable in the environment than enterovirus. A study comparing the stability of enterovirus 70 and human adenovirus type 19 under

various conditions observed that the drying of these agents, at room temperature resulted in a 5-Log_{10} reduction of enterovirus 70 and a less than 1-Log_{10} reduction of adenovirus after 11 days under identical conditions (Hara et al., 1990). The stability of adenovirus is also supported by Mahl and Sadler (1975) who compared the survival of several viruses at different temperatures and relative humidity values. They observed that adenovirus type 2 survived longer than poliovirus 2, vaccinia virus, coxsackievirus B3, and herpes virus, at relative humidity values ranging from 3 to 96 % at temperatures of 25 to 37° C.

Evidence of increased thermal stability of adenoviruses compared to polio 1 was obtained in an experiment where enteric adenovirus 40 (Ead 40) and polio 1 were incubated at 50, 65, and 80° C in a solution of phosphate buffered saline. Incubation at 50° C for 6 minutes resulted in a 0.88 Log_{10} reduction of polio 1 whereas Ead 40 reduction was only 0.2 Log_{10}. At 65° C polio was reduced 2.5 Log_{10} in 10 seconds while Ead 40 decreased only 1 Log_{10}. When the experiment was conducted at 80° C, polio decreased 4 Log_{10} in 30 seconds compared to Ead 40 which decreased only 1.8 Log_{10} (Enriquez et al., 1995).

In addition to thermal stability, adenoviruses show resistance to high and low pH. Experiments to evaluate adenovirus stability at pH values of 3.5, 9.5, and 10.0 in 0.1M glycine buffer, showed no reduction in infectivity after exposure for 45 minutes. At a pH value of 10.5, a 2.7 Log_{10} reduction of the original titer was observed (Enriquez et al., 1995). A previous study contradicting these results by Fields and Metcalf (1975) reported rapid inactivation of adenovirus type 5 at a pH value of 10.0.

Methods of Sludge Treatment and Their Effectiveness in Pathogen Removal

Sludge from domestic sewage treatment needs to be treated to reduce organic matter and water content, remove unpleasant odors, and reduce the concentration of pathogens to proposed USEPA regulations. There are four basic methods of sludge treatment, each with its own advantages and disadvantages. These processes include mesophilic or thermophilic anaerobic digestion, aerobic digestion, composting, and lime stabilization. Facilities may use a combination of these methods to achieve the desired level of treatment.

Anaerobic digestion can be mesophilic (temperature range of 25° to 40° C) or thermophilic (50° to 65° C) (Bitton, 1994). High rate reactors are commonly used to mix the sludge under anaerobic conditions followed by heating to either mesophilic or thermophilic temperatures. Low rate reactors allow the sludge to settle, and reactions proceed anaerobically for 30 to 60 days. Larger facilities use anaerobic digestion to treat sludge because methane gas produced during the process is recovered and used to supply energy to the facility (Bitton, 1980). An advantage of anaerobic digestion is it does not require an input of air required in aerobic digestion which is an expensive feature (Pederson, 1983). Reduction of pathogenic microorganisms by anaerobic digestion is dependent on both temperature and time. Digestion at thermophilic conditions for longer detention times increase the reduction of potential pathogens (Ward and Ashely, 1977).

The use of aerobic digestion to treat sewage sludge is common in smaller facilities (Bitton, 1980). Aerobic digestion temperatures are usually mesophilic (25° to 40° C) with a mean retention time of 10 to 20 days. Air must be pumped into the reaction tanks

increasing the costs due to the energy input. Pathogen reduction may also be limited (Pederson, 1983). The conversion of organic matter to carbon dioxide and water leads to the reduction of bacteria from nutrient (carbon source) deprivation.

Another means of sludge treatment is mesophilic composting. Liquid sludge is mixed with a bulking agent such as wood chips, dry compost or municipal refuse. Naturally occurring microorganisms in the pile increase the temperature inside up to 60° C (Bitton, 1994) from oxidation by microorganisms of substrates in the sludge (Atlas and Bartha, 1993). After nutrient deprivation is complete, the pile cools to ambient temperatures and the organic matter of the sludge has been mineralized to carbon dioxide and water or transformed to stable humic like substances. Unfortunately temperatures at the outer edge of the pile are not lethal to microorganisms and the pile can be re-inoculated during turning.

The fourth method for treating sludge is lime stabilization (Pederson, 1983). Liquid sewage sludge is mixed with a sufficient amount of lime (in the form of $Ca(OH)_2$ or CaO) to raise the pH to 12.0 or above for at least two hours. At this pH the NH_4^+ moiety is deprotonated resulting in the production of ammonia gas. The combination of pH and ammonia gas can reduce enteroviruses by 4 orders of magnitude (Sattar et al., 1976).

An overview of microbial reduction by the various sludge treatment processes is listed in Table 2.2. Estimated concentration of viruses after sludge digestion are presented in Table 2.3. Note that despite a 1 to 2 Log_{10} reduction in pathogen numbers,

significant concentration of these pathogens may persist after sludge treatment (Pepper and Gerba, 1989).

TABLE 2.2: An Overview of Microbial Reduction During Sludge Treatment as Normally Practiced[1]

Treatment	Reduction[2]		
	Bacteria	**Viruses**	**Parasites**
Anaerobic digestion	1-2	1	0
Aerobic digestion	1-2	1	0
Composting	2-3	2-3	2-3
Lime stabilization	2-3	3	0

[1]Adapted from Straub, 1993.

[2]Scale:

0= <0.5 orders of magnitude (<10% reduction)

1= 0.5 to 2 orders of magnitude (99% reduction)

2= 2 to 4 orders of magnitude (99.9% reduction)

3= >4 orders of magnitude (99.99% reduction)

TABLE 2.3: Virus Concentration in Digested Sludge[1]

Organism	Type of Treatment	
	Anaerobic	Aerobic
	(per g dry wgt)	
Enteroviruses	0.2-210	0-260
Rotaviruses	14-485	ND[2]

[1]Adapted from Straub, 1993.

[2]ND = not determined

Cell Lines Used for Virus Detection in Environmental Samples

Environmental virology has focused on the use of cell culture for viral detection and enumeration. One of the concerns in monitoring for waterborne viruses is false negatives from the lack of sensitivity in concentration procedures and the subsequent viral assay (Dahling et al., 1984; Keswick and Gerba, 1980). Since the late 1980's there has been a movement toward improving the sensitivity in cell culture which hopefully will lead to standardized cell culture procedures (Dahling, 1991). The factors influencing virus detection in cell culture are too numerous to list and beyond the scope of this manuscript. An in-depth overview of all the factors for enteric viruses in cell culture is discussed by Dahling (1991).

Morris and Waite (1980) evaluated Vero, BGM and RD continuous cell lines for virus recovery from river waters. Their results showed increased virus recovery using BGM cell followed by RD cells. Vero cells proved to be relatively inefficient. Morris (1985) further assessed ten continuous cell lines (five of human origin, five of simian derivation) including BGM, MA-104, Vero, human embryonic jejunum and normal human amnion for enterovirus detection. Morris demonstrated that detection of naturally occuring enteroviruses was most efficient when BGM cells were used with 82 % of samples being positive followed by RD cells with 73 %.

Table 2.4 lists human viruses that most effect the human population and the cell culture line used to detect them. It should be noted that although many of these agents will grow and plaque in cell culture, some do not.

TABLE 2.4: Some Human Viruses and Their Detectabillity in Cell Culture[1]

Virus Group	Number of serotypes	Virus type	Culturable in[2]
Adenovirus	49	1-33, 39	HEK
		1-18, 24-33, 38, 41	HeLa
		36, 37	HEL
		34, 35, 41	Hep-2
		40, 41	MkK, PFL/PRF/5
Poliovirus	3		MkK
Echovirus	32		MkK
Coxsackie A	23	1, 4-6, 17, 19, 22	sM
		2, 3, 8, 10-15, 18, 20, 21, 24	Ham
		3, 5, 7, 9, 16	MkK
Coxsackie B	6		MkK
Enterovirus	4	68, 69, 71,	MkK
		70	HEK
Reovirus	3		MkK
Rotavirus	4		MkK
Hepatitis A	1		MkK, MA-104
Astrovirus	5		CaCo-2
Calicivirus	2		None
Coronavirus	1		None
Norwalk, Norwalk-like and related agents	9		None

[1]Modified from Dahling (1991).

[2]HEK = human embryo kidney; HeLa = human cervical carcinoma cells; HEL = human embryonic lung; HEp-2 = human laryngeal tumor cells; MA-104 = monkey kidney; MkK = monkey kidney primary and continous; sM = suckling mice; Ham = human amnion; CaCo-2 = human colon carcinoma; PLC/PRF/5 = primary human hepatocellular carcinoma.

Table 2.5 lists the most commonly used cell lines for enteric virus isolation from environmental samples. Disagreement exists as to the particular viruses that are supported by these cells lines probably due to different virus isolation patterns from the type of sample under test. For environmental samples, where mixed groups of viruses are encountered, some viruses will outgrow others leading to a misrepresentation of the actual amount present (Dahling and Wright, 1986). It is impractical for a laboratory to maintain more than three or four cell lines concurrently therefore a list of recommended cell lines is presented in Table 2.6.

TABLE 2.5: Sensitivity of Various Cell Lines to the Major Enterovirus Groups in Environmental Samples

Sample type	Cell line[3]	Enterovirus group tested[2]			
		Poliovirus	Echovirus	Coxsackie type A	Coxsackie type B
Environmental lab virus	BGM	+	+	+	+
	MA-104	+	+	+	+
	RD	+	+	+	+
	HEp-2	+	-	+	+
	Vero	+	±	+	+
	L132	+	-	±	±
	HeLa	+	-	±	±
	MDBK	-	-	-	-
	PAGMK	+	+	+	+
	PRhMK	+	+	+	+
Environmental	PRhMK	+	+	-	-
	MFK	+	+	-	+
	BGM	+	+	+	+
	HFDK	+	±	+	-
	RD	+	+	+	-
	FL	+	+	+	+
	407	+	+	+	+
	D6	+	+	+	+
	CC	+	+	+	+
	CL	+	+	+	+
	LLC	+	+	+	+
	Vero	+	+	+	+
	MA-104	+	+	+	+
	HeLa	+	±	-	±
	KB	+	±	-	±
	HEp-2	+	±	-	+
	MRC-5	+	+	+	-

[1]Modified from Dahling (1991).

[2] + = posititve reaction; - = negative reaction; ± = reaction some of the time

[3]Cell lines tested: BGM = Buffalo green monkey; PAGMK = primary African green monkey; PRhMK = primary Rhesus monkey kidney; MFK = fetal Rhesus monkey kidney; HDFK = human fetal kidney; RD = human rhabdomyosarcoma; FL = human aminon; 407 = human embryonic jejunum; D6 = Detroit-6 human amnion; CC = Chang conjunctive 'D' cells; CL = Chimpanzee liver; LLC = Rhesus monkey kidney; Vero = monkey kidney; MA-104 = monkey kidney; HEL = human embryonic lung; HEp-2 = human carcinoma; L-132 = human embryonic lung; HeLa = human cervical carcinoma; MDBK = bovine kidney; MRC-5 = human embryonic lung; KB = human carcinoma.

TABLE 2.6: Recommended Cell Lines for Enterovirus Isolation in Envrionmental Samples[1].

	Virus group				
Cell line[2]	**Poliovirus**	**Echovirus**	**Coxsackie A**	**Coxsackie B**	**Reovirus**
BGM	+	+	+	+	-
RD	+	+	+	-	-
MRC-5	+	+	+	-	-
MDBK	-	-	-	-	+

[1]Modified from Dahling (1991).

[2]Cell lines: BGM = Buffalo green monkey; RD = human rhabdomyosarcoma; MRC-5 = human embryonic lung; MDBK = bovine kidney.

Molecular Detection Methods for Viruses

The emergence of recombinant DNA technology has resulted in new and more sensitive detection assays with improved specificity and selectivity. *In vitro* amplification of DNA using the polymerase chain reaction (PCR) or RNA using reverse transcriptase-PCR (RT-PCR) allows for improved detection of viral pathogens in environmental samples (Abbaszadegan et al., 1993). PCR is the *in vitro* enzymatic amplification of target nucleic acids directed by a specific pair of oligonucletide primers. Primers bind to denatured strands of DNA or (for RT-PCR) cDNA at a complementary site and facilitate a DNA-polymerase to extend the nucleic acid to a specific size and sequence (Katner, 1998). PCR assays have been applied to the detection of enteroviruses and adenoviruses in clinical (Rotbart, 1990; Allard et al., 1990) and environmental samples (Abbaszadegan et al., 1993; Girones et al., 1993).

The advantages of PCR for the detection of viruses are: 1) the cost is a fraction compared to a cell culture assay; 2) detection of viruses is reduced from 4 weeks to less than 24 hours; 3) specific strains of viruses are identifiable without the need of serology; and 4) viruses that do not grow in cell culture can be detected. This is especially important for Norwalk and Calciviruses which currently cannot be grown in cell culture. Some of the disadvantages to PCR when compared to cell culture are: 1) PCR is non quantifiable; 2) PCR does not establish if the virus is infectious; and 3) PCR only assays small volumes (5-10 L equivalent volumes for PCR compared to >2000 L for cell culture) (Katner, 1998).

Multiplex Polymerase Chain Reaction (M-PCR)

Multiplex polymerase chain reaction (M-PCR) is the simultaneous detection and amplification of more than one target DNA sequence. This is accomplished in the same manner as PCR except that two or more sets of oligonucletide primers are used to target different sequences of DNA. Enzymatic amplification then produces two or more distinguishable amplicons. Most of the initial work with M-PCR addressed the diagnosis of inherited genetic disease; however; more work in the diagnosis of infectious agents has been reported (Mahony and Chernesky, 1995).

The major advantage to M-PCR is the ability to detect more than one agent in a single test. In the clinical diagnostic laboratory M-PCR can be used to differentiate between clinically indistinguishable episodes of disease (Jackson et al., 1996). The ability to differentiate between or detect more than one viral agent in a single test has major potential in environmental virology where samples often have mixed groups of viruses (Dahling and Wright, 1986). The drawbacks to M-PCR are similar to those for PCR. Of particular concern when developing M-PCR is the concentration of each set of primers and the optimal annealing temperature for these sets. The primer concentration in M-PCR is generally not 1:1 due to the ability of primer sets to out-compete each other for polymerase enzyme and free Mg^{+2}. Optimal annealing temperature for primer sets is usually different; however, it is recommended that the theoretical melting temperature (temperature at which 50% of the primer DNA has disassociated from its complement) or T_m of both primer sets have no more than a 10°C difference. Therefore a compromise

annealing temperature, where each primer set anneals and undergoes enzymatic amplification, must be determined empirically.

CHAPTER 3 PRESENT STUDY

The objectives of this study are: 1) the comparison of the BGM and CaCo-2 continuos cell lines for the recovery of enteric viruses in mesophilic treated sewage sludge; 2) the isolation of adenovirus in treated sludge; 3) the evaluation of concentration methods for adenovirus in treated sludge; and 4) the development of a multiplex polymerase chain reaction (M-PCR) to simultaneously detect all human adenoviruses and specifically adenovirus types 40 and 41.

CHAPTER 4 MATERIALS AND METHODS

Experimental Overview

Undigested and anaerobically digested sludge samples were collected every two months for one year. Viruses were eluted and reconcentrated using standard methods (ASTM, 1993). Reconcentrate was assayed on BGM and CaCo-2 continuous cell lines for enteroviruses and again on CaCo-2 cells for adenoviruses. The identity of adenovirus isolates in digested sludge was confirmed using PCR. M-PCR was developed to detect adenovirus and the F-type adenovirus 40 and 41.

Facilities Description

Pima County's Ina Road Wastewater Treatment Plant (IRWTP) is located on the west side of Tucson, AZ in the Sonoran desert. The facilities utilize an initial grit chamber followed by passage of the sewage into the primary settling tanks. Effluent then passes over aerobic biotowers followed by secondary settling tanks. The effluent is chlorinated before being discharged into the Santa Cruz River. Sludge collected from the primary and secondary settling tanks undergo mesophilic anaerobic digestion for 2-3 weeks at 30° C. For the raw and mesophilic sewage sludge samples the mean percent solids (dry weight basis) were 4.3 and 2.1 %, respectively. The mean retention time for sludge in the mesophilic digestor was 18 days.

Sample Collection and Elution

Undigested and anaerobically digested sewage samples were collected from the Ina Road Wastewater Treatment Plant, Tucson Arizona, in sterile 1-L polypropylene bottles (Nalgene; Nalge Co., Rochester, NY); transported to the laboratory on ice, and stored at 4° C until processed. Samples were processed within 48 hours post arrival in the laboratory by the beef extract elution and concentration procedure described in the American Society for Testing and Methods (1993). A 500 mL aliquot of the liquid sludge sample was eluted by the addition of $AlCl_3$ to a final concentration of 0.0005M and the pH adjusted to 3.5 by drop-wise addition of 1 N HCl. The mixture was stirred for 30 min, decanted into sterile 250-mL centrifuge bottles (Nalgene; Nalge Co., Rochester, NY) and centrifuged for 30 min at 15,300 x g (JA-14 rotor; Spinco Division, Beckman Instruments, Palo Alto, CA). The resulting pellet was resuspended by constant stirring for 30 min at room temperature using 500 mL of sterile 3 % beef extract (Beef Extract V; BBL Microbiology Systems, Cockeysville, MD) buffered to pH 9.5 ± 0.1 with 7.3 g / L Na_2HPO_4 (EM Science, EM Industries Inc., Cherry Hill, NJ) and 1.0 g / L citric acid (Mallinckrodt, St. Louis, MO). The suspension was then decanted into sterile 250-mL centrifuge bottles and centrifuged at 15,300 x g for 30 min. The supernatant was collected and concentrated by acid flocculation of the solution proteins by lowering to pH 3.5 ± 0.1 by the drop-wise addition of 1.0 N HCl. The suspension was then decanted into sterile 250-mL centrifuge bottles and centrifuged at 15,300 x g for 10 min. The pelleted proteins were then dissolved in 30 mL of 0.15 M Na_2HPO_4 and adjusted to pH 7.2 ±0.1.

To reduce potential biotic and abiotic contaminants, the sample was divided evenly in two 50-mL centrifuge tubes (Costar Corporation, Cambridge MA) and extracted by adding an equal volume of Freon (1,1,2 trichlorotrifluroethane; EM Science, EM Industries Inc. Cherry Hill, NJ) mixed until an emulsion formed and centrifuged at 2500 rpm for 30 min (IEC Centra-7 centrifuge, International Equipment Co., Needham Heights, MA). The aqueous top layer was subsequently removed and treated with kanamycin, gentamycin and antibiotic/antimicotic (BRL Life Technologies, Gaithersburg, MD) to reduce bacterial and fungal contamination. Samples were stored at -80° C until cell culture and PCR assays were performed.

Cell Culture Assays for Enterovirus and Adenovirus in Sludge

Detection and enumeration of enterovirus and adenovirus in raw and treated sludge samples were determined by a most probable number (MPN) as described in *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association, 1995). Continuous cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville MD) and used between passage numbers 97-114 and 123-166 for CaCo-2 and BGM cell lines respectively. For enterovirus and adenovirus assays, Buffalo Green Monkey cells (BGM) and Human Colon Carcinoma 2 cells (CaCo-2) respectively were grown for 5 to 7 days at 37° C in 75 cm^2 tissue culture flasks (Corning; Corning, NY) until a confluent monolayer was formed. The growth media for the cells lines consisted of 1x minimal essential media (BRL Life Technologies, Gaithersburg, MD) 200 mM glutamine and antibiotics (kanamycin [100 µg/mL], antibiotic / antimicotic [100µg/mL] BRL Life Technologies, Gaithersburg, MD) buffered to pH 7.2 with 7.5 % sodium bicarbonate and 1 M HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; Research Organics, Cleveland, OH). For BGM and CaCo-2 cell lines the growth media described above was supplemented with 5 % and 10 % fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), respectively. Growth media for all flasks was decanted and the cell monolayer washed once with 4 mL of sterile tris buffered saline (Trizma base; Sigma Chemical Co., St. Louis, MO.) and decanted. Prior to inoculation the thawed concentrate was filter-

sterilized using a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI.) and diluted with 1x MEM containing 2 % FBS.

For the adenovirus assays, diluent was supplemented with guanidine HCl to a final concentration of 1.05 mM in an effort to inhibit any enteroviruses present in the sample (Hurst et al., 1988). The flasks were inoculated with the appropriate dilutions of the concentrate and allowed to adsorb at 37° C for 30 min. After adsorption, 20 mL of maintenance media consisting of 1x MEM (previously described) with 2 % FBS was added to each flask. For adenovirus assays, the maintenance media was supplemented with guanidine HCl to a final concentration of 1.05 mM. All flasks were incubated at 37° C and observed daily for 14 days for cytopathogenic effect (CPE) indicative of viral infection. All flasks for both enterovirus and adenovirus assays showing CPE were subject to confirmation by freeze/thawing the cells, filter sterilizing with a 0.2 μm-pore size filter and inoculating 0.1 mL of the lysate on fresh cells. CPE within 2 to 7 days post inoculation was confirmation of the presence of viruses.

The most probable number method used to determine the concentration of viruses in raw and treated sludge samples is given in Equation 1 (Standard Methods for the Examination of Water and Wastewater):

Equation 1

$$MPN/mL = \frac{\text{number of positive flasks}}{\sqrt{(\text{mL of sample in negative flasks}) \times (\text{mL of sample in all flasks})}}$$

This formula was used for both enterovirus and adenovirus assays to estimate the number of viruses detected in each cell line. For adenoviruses all flasks showing CPE were subjected to M-PCR to confirm the presence of adenoviruses.

Adenovirus primer sequences used in this study were described by Girones et al. (1993). The design of the adenovirus primers is based on the adenovirus 2 hexon polypeptide which is highly conserved amongst adenovirus subgenus A-F (Allard et al., 1990). The specific sequence for the primers are:

hex AA1885 5′-GCC GCA GTG GTC TTA CAT GCA CAT C-3′

hex AA1913 5-′CAG CAC GCC GCG GAT GTC AAA GT-3′

hex AA1885 and hex AA1913 refer to the sequence position along the adenovirus 2 hexon region for upstream and downstream primers, respectively. PCR amplification using theses primers results in a 300 base pair (bp) amplicon. This first set of primers was combined (for M-PCR) with primers developed by Tiemessen and Marietha (1996) which are specific for the long tail fiber gene highly conserved in enteric adenovirus type 40 and 41 only. The specific sequence of these primers are:

K402 5′-CAC TTA ATG CTG ACA CG-3′

K403 5′-ACT GGA TAG AGC TAG CG-3′

The amplification product resulting from these primers is 152 bp long.

Presumptive adenovirus tissue culture isolates were subjected to one cycle of freeze/thaw to release virions, followed by Freon extraction of a 500 µl aliquot of the

supernatant to remove cellular debris. The upper aqueous layer was decanted and used in M-PCR.

Multiplex Polymerase Chain Reaction (M-PCR)

To determine the optimal conditions for M-PCR several concentrations of primers and PCR reagents were tested using PCR opti-prime kit (Stratagene, La Jolla, CA.), in conjunction with varying primer annealing temperatures (see results section). The final concentrations for 100 μL reactions were: 10mM Tris HCl (pH 8.3), 1.5mM $MgCl_2$ and 25mM KCl, (Stratgene buffer no. 1) primers hexAA1885 and AA1913 at 100 pmol each, primers K402 and K403 at 50 pmol each and 2.5 U Taq DNA polymerase (Perkin Elmer, Branchburg, NJ.). Thin walled reaction tubes (Perkin Elmer, Branchburg, NJ.) contained 10 μl of sample and were carried out in a Perkin Elmer model 2400 Thermocycler (Perkin Elmer, Branchburg, NJ.) using the following cycling profile: One cycle for 25 min at 94° C to denature the viral capsid, followed by 30 cycles primer annealing for 90 sec at 60° C, extension for 90 sec at 72° C and denaturing for 30 sec at 94° C. A final extension cycle of 7 min at 72° C was also performed immediately followed by a 4° C hold cycle. The reaction conditions described here were used for cell culture isolates as well as adenovirus seeded sludge samples.

Detection of Amplified Product

Amplification products (20 μl/sample) were separated on a 2 % agarose gel (SeaKem agarose; FMC Bioproducts, Rockland, ME) via submarine gel electrophoresis

for 1.5 hr at 50 V/cm in 0.5x tris-borate-EDTA buffer (BioRad submarine gel electrophoresis box; BioRad, Melville, NY). The gel was stained with ethidium bromide and visualized using ultraviolet transillumination and photography (UV transilluminator and camera; Fotodyne, New Berlin, WI)

Efficiency Testing for Adenovirus Recovery

To investigate the recovery of adenovirus from treated sludge, samples were collected as previously described and seeded with $1x10^6$ $TCID_{50}$ of adenovirus type 2. Sample elution and reconcentration were performed as previously described. Calculation of virus titer was performed using the $TCID_{50}$ method in CaCo-2 cells.

CHAPTER 5 RESLUTS

Samples were taken from the Ina Road Waste Water Treatment Plant in Pima County, Tucson AZ for the isolation of adenovirus in treated sludge. For the comparison of BGM and CaCo-2 cell lines samples described above were used in conjunction with samples shipped to the laboratory from water treatment facilities in Phoenix AZ. These samples were processed by beef extract elution and organic precipitation (USEPA, 1996).

Adenovirus Isolation in Treated Sewage Sludge

Adenoviruses demonstrated CPE in 3 of 47 cell culture flasks (Table 5.1). The 3 adenovirus isolates were found in mesophilic-treated sewage sludge. The remaining 44 flasks were identified as enteroviruses by semi nested PCR.

Recovery Efficiency for Adenovirus in Sewage Sludge

The mean recovery for adenovirus in seeded mesophilic digested sewage sludge was 88 % (SD 2.9; n=3) with a range of 85-90 % (Table 5.2). The mean initial and final titers were 9.3×10^5 and 1.0×10^5 respectively.

Enterovirus Inactivation in Raw and Treated Sewage Sludge

Enteroviruses were present in 100 % of raw sewage samples (Table 5.3). The mean concentration in raw sewage was 31.4 MPN / 4g dry weight with a range of 4.6-67.5 (SD = 24.3, n = 5). In treated mesophilic sewage sludge one sample was positive for enteroviruses (Table 5.3) with a concentration of 1.4 MPN / 4g dry weight. All other

TABLE 5.1: Adenovirus in Sewage and Treated Sewage Sludge Using the CaCo-2 Cell Line[1]

Sample date and type[2]	**No. of cell culture flasks[3]**	**No. of positive cell culture flasks**	**PCR results[4]**
12/96 R	12	0	negative
12/96 M	12	0	negative
2/97 R	5	5	Ent.
2/97 M	5	0	negative
4/97 R	9	4	Ent.
4/97 M	9	3	Adeno.
7/97 R	20	19	Ent.
7/97 M	7	2	Ent.
9/97 R	10	10	Ent
9/97 M	10	4	Ent.

[1]All samples were obtained from the Ina Road Treatment Facility, Tucson AZ.

[2]R = raw sewage sludge; M = mesophilic digested sludge.

[3]Varying flask numbers were used as to eliminate toxicity.

[4]Ent. = enterovirus PCR positive; Adeno = adenovirus PCR positive

TABLE 5.2: Recovery Efficiencies for Adenovirus in Seeded Mesophilic Treated Sewage Sludge[1]

	Initial titer Calculated by $TCID_{50}$	Final titer	% recovery
Trial A	$2x10^5$	$2x10^4$	90
Trial B	$2x10^5$	$9x10^4$	85
Trial C	$2x10^6$	$2x10^5$	90
mean	$9.3x10^5$	$1.0x10^5$	88
SD			2.9
n	3	3	3

[1]Virus titers were performed using CaCo-2 cells and enumerated using the Reed-Muench formula

TABLE 5.3: Enterovirus Inactivation in Raw and Mesophilic Digested Sewage Sludge

Sample date	Raw sewage	Mesophilic sewage	Removal %
	MPN / 4g dry weight[1,2]		
Dec. 96	4.6	<0.2	>95.6
Feb. 97	19.9	<0.2	>98.9
Apr. 97	22.4	<0.2	>99.1
Jul. 97	67.5	<0.2	>99.7
Oct. 97	42.7	1.4	96.7
Mean	31.4	-	98
SD	24.3	-	1.7
n = 5			

[1]Most probable number / 4g dry weight.

[2]Samples with no CPE were reported as <0.2 MPN / 4g dry weight.

treated sludge samples negative for CPE were reported as < 0.2 MPN / 4g dry weight. The mean percent removal was 98 % with a range of 95.6-99.7 % (SD = 1.7, n = 5), (Table 5.3).

Comparison of BGM and CaCo-2 Continuous Cell Lines for Enteric Viruses

A overview of BGM and CaCo-2 cell lines for the recovery of enteric viruses in mesophilic treated sewage sludge (MTS) and wastewater effluent (WWE) samples is presented in Table 5.4. The data was reported as MPN / mL of reconcentrate. Samples that were negative for CPE are reported as <0.2 MPN / mL.

A comparison of BGM and CaCo-2 cell lines for the recovery of enteroviruses in mesophilic treated sludge is presented in Table 5.5. Results are reported as MPN / 4g dry weight. Semi-nested PCR identified the isolates as enteroviruses (data not shown). Samples that were negative for CPE are reported as <0.2 MPN / mL.

Comparison of BGM and CaCo-2 cell lines for the recovery of enteric viruses in secondary treated sewage disinfected with chlorine is presented in Table 5.6. The CaCo-2 cell line showed an average increased recovery of 11.8 x (range = 1.6-15 x) for enteric viruses over BGM cells. None of the samples compared were negative for CPE in either BGM or CaCo-2 cells. Positive CPE cell culture flasks (CaCo-2 cells) were passed on BGM cells to confirm enteroviruses.

TABLE 5.4: Comparison of BGM and CaCo-2 Cell Lines for the Detection of Enteric Viruses in Environmental Samples

	Cell culture lines[2]	
	BGM	CaCo-2
Sample type[1]	Reported as MPN/mL[3]	
RSS	124.9	513.4
MTS	<0.2	4.7
MTS	3.4	41.2
MTS	<0.2	<0.2
MTS	<0.2	13.5
MTS	<0.2	<0.2
MTS	<0.2	<0.2
WWE	3.3	44.1
WWE	3.8	50.4
WWE	3.4	50.4
WWE	6.1	84.3
WWE	2.4	5.1

[1]RSS = raw sewage sludge; MTS = mesophilic treated sludge; WWE = waste water effluent.

[2]BGM = Buffalo green monkey; CaCo-2 = human colon carcinoma.

[3]MPN/mL = most probable number/mL.

TABLE 5.5: Comparison of BGM and CaCo-2 Cell Lines for the Detection of Enteroviruses in Mesophilic Treated Sewage Sludge

Sample	Cell culture lines[1]		
	BGM	CaCo-2	
	Reported as MPN / 4 g dry weight[2]		PCR Results[3]
1	<0.2	1.5	Enterovirus
2	1.4	16.5	Enterovirus
3	<0.2	<0.2	-
4	<0.2	5.4	Enterovirus
5	<0.2	<0.2	-
6	<0.2	<0.2	-

[1]BGM = Buffalo green monkey; CaCo-2 = human colon carcinoma.

[2]MPN/mL = most probable number/4g dry weight.

[3]Enterovirus = PCR positive for enterovirus in CaCo-2 cells

TABLE 5.6: Comparison of BGM and CaCo-2 Cell Lines for the Detection of Enteric Viruses in Secondary Treated Sewage Disinfected With Chlorine

	Cell culture lines[1] Reported as MPN / 10L[2]	
Sample	**BGM**	**CaCo-2**
1	0.08	1.2
2	0.10	1.3
3	0.15	2.2
4	0.16	2.2
5	0.06	0.1

[1]BGM = Buffalo green monkey; CaCo-2 = Human colon carcinoma.

[2]MPN / 10L = most probable number / 10 L.

Multiplex Polymerase Chain Reaction

Virus stocks were grown in a human colon carcinoma (CaCo-2) continuous cell line and titered via by the $TCID_{50}$ method. Dilutions of the virus stock were used directly in the development of M-PCR. The amplicon from the primers were for the hexon gene region (hexAA1885 and 1913) 308 bp and for the long fiber gene (K402 and 403) 152 bp. Gel electrophoresis of the optimal PCR buffer concentrations are presented in Figure 5.1. Optimal primer concentrations and annealing temperature were determined by trial and error in a PCR thermocycler (Perkin Elmer model 2400) (data not shown). Optimal final reaction concentrations were 10mM Tris HCl (pH8.3), 1.5mM $MgCl_2$ 25mM KCl, 2.5U taq DNA polymerase with 100 pmol each hex primer and 50 pmol each long fiber primer. Reaction conditions were: an initial denaturation step of 94° C for 25 min, followed by 35 cycles of annealing at 60° C for 90 sec, extension at 72° C for 90 sec and denaturing at 94° C for 30 sec.

Sensitivity of the M-PCR to single and mixed stocks of adenovirus type 2 and enteric adenovirus (Ead) type 40 are presented in Table 5.7. With a single virus type the sensitivity was as little an 10^0 $TCID_{50}$, whereas with mixed viruses the sensitivity was 10^2 $TCID_{50}$.

To test the ability of M-PCR amplification in treated sewage sludge, mesophilic-treated sludge was seeded with 6.4×10^3 $TCID_{50}$ of adenovirus type 2 and 2.0×10^2 $TCID_{50}$ of Ead 41 and subjected to three different DNA extraction protocols in an effort

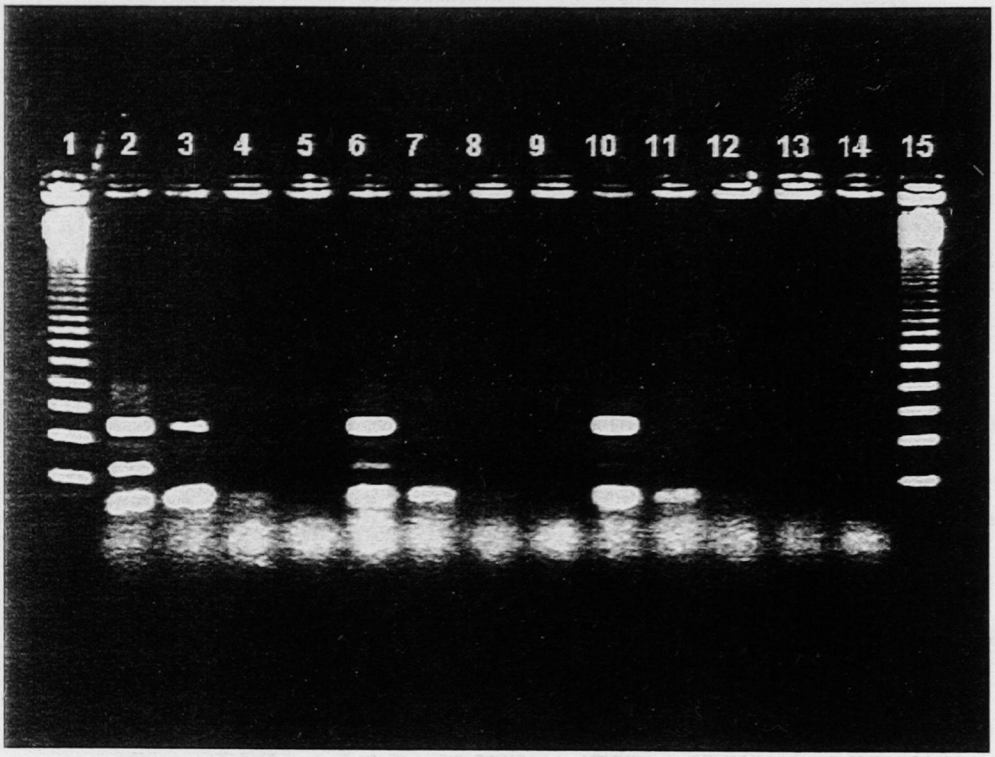

FIGURE 5.1: DETERMINATION OF THE OPTIMAL PCR BUFFER CONCENTRATIONS

Gel electrophoresis of M-PCR products to determine the optimal buffer concentrations. A 2% agarose gel was run for 1.5 hrs. at 50 v, stained with ethidium bromide and photographed using UV transillumination. Lane identification: lanes 1& 15, 123 bp DNA ladder; lanes 2-13, Opti-Prime buffers #1 through #12; lane 14, negative control.

TABLE 5.7: Sensitivity of M-PCR to Single and Mixed Dilutions of Adenovirus.

Virus[2]	Dilution[1]				
	10^3	10^2	10^1	10^0	10^{-1}
	Band intensity[3]				
Ad-2 only	+++	+++	++	+	-
Ead-41 only	+++	+++	++	+	-
Ad-2 and Ead-41 mixed[4]	+++	++	-	-	-

[1]Virus stocks titered by the $TCID_{50}$ method.

[2]Ad-2 = adenovirus type 2; Ead-41 = enteric adenovirus type 41

[3]Comparative scale of band intensity: - = no band, += faint band, ++= medium band +++= bright band.

[4]Equal amounts of Ad-2 and Ead-41 mixture

to remove PCR inhibiting substances. Briefly, the first protocol involved heating the sample to 94° C for 25 min to denature the viral capsid and release the DNA (Allard et al., 1990). The second protocol was a universal DNA extraction using guanidine thiocynate/phenol (GPT) extraction and ethanol precipitation (Gurpreet et al., 1995). The third protocol was a QIAamp blood and tissue DNA purification kit (Qiagen, Valencia CA) blood and body fluid DNA extraction protocol steps 1-9.

Results of M-PCR amplification using these three methods are presented in Table 5.8. All three methods of viral DNA extraction in seeded sludge reconcentrate produced amplicons of 300 bp for Ad-2, but only heat denaturation and GPT extraction produced 152 bp amplicon for Ead-40. In sludge reconcentrate seeded with a combination of Ad-2 and Ead-41, all three methods failed to produced either amplicon (300 and 152 bp) simultaneously

TABLE 5.8: DNA Extraction Protocols and M-PCR Results for Adenovirus Seeded Mesophilic Treated Sewage Sludge Reconcentrate

	DNA extraction protocol		
M-PCR product[1]	**Heat denaturation**	**Guanidine/phenol**	**Qiagen**
Ad-2	+	+	+
Ead-40	+	+	-
Ad-2/Ead-40	-	-	-

[1]Reconcentrate was seeded with 10^3 type 2 and 10^2 type 40 adenovirus ($TCID_{50}$).

Statistical Analysis

To compare the sensitivity of BGM and CaCo-2 cell lines for the recovery of enteric viruses and enteroviruses in environmental samples, the data was initially tested for a normal distribution by plotting (data not shown). The data was not normally distributed thus failing the assumptions necessary for parametric statistical analysis (i.e. a student *t*-test). The statistical analysis that best fitted the data given the small sample size and distribution was the Wilcoxon rank-sum test. This is a non-parametric test which places all the data in increasing order while retaining group identity (i.e. groups CaCo-2 and BGM). The sum of each group for CaCo-2 larger than BGM is evaluated so that the probability of occurring by chance is small (<0.05).

CHAPTER 6 DISCUSSION

Adenovirus Isolates in Treated Sludge

The results suggest that adenovirus can survive mesophilic sludge treatment (Table 5.1). However their occurrence in raw sewage sludge was not determined possibly due to the inability of guanidine HCl to suppress wild type enteroviruses. Hurst et al. (1998) showed only a 2-7 $\log_{10}$ reduction of wild type enteroviruses in the presence of guanidine. This combined with the increased sensitivity of CaCo-2 cells (Pinto et al., 1995) may have let the faster growing enteroviruses establish CPE. Many of the isolates in CaCo-2 cells were identified by PCR as enteroviruses.

Another possibility is the lack of adenovirus in the sample. Girones et al. (1993) found only 5 adenovirus isolates in 20 sewage samples. This is supported by Tani et al. (1995) who over five years of weekly sampling of river water polluted with sewage from a city of 300,000 people, found only 20 adenovirus isolates with no apparent seasonal preference.

The average recovery efficiency for adenovirus was determined to be 88 % in this study (Table 5.2) suggesting that the concentration method works for this particular virus. Similar results were obtained by Sattar and Westwood (1976) and Hurst et al. (1978).

Previous studies contradicting the low number of adenoviruses have been reported. Irving and Smith (1981) found adenoviruses in 96, 88 and 88 % of raw sewage, primary and secondary effluent respectively. They also suggest that adenoviruses (along with reoviruses) are more likely to survive conventional sewage treatment than

enteroviruses. Krikelis et al. (1985) had 100 % (n=24) of raw sewage samples positive for adenoviruses with a mean concentration of 280 cytopathogenic units / L (CPU / L) and Williams and Hurst (1988) reported a mean concentration of 10,800 fluorescent focus-forming units / L (FFU / L) in raw primary sewage sludge.

The results of this study failed to establish a seasonal occurrence of adenovirus in raw and treated sewage sludge. Previous studies suggest both a low and a high level of adenovirus in wastewater sludges suggesting that their occurrence in sludges is dependent on yet unidentified factors.

Enterovirus Inactivation in Raw and Mesophilic Sewage Sludge

The results of enterovirus removal at the treatment facility are similar to those reported by Straub et al. (1994). The average reduction of enteroviruses after mesophilic digestion was 98 % corresponding to a 2 $\log_{10}$ reduction (Table 5.3). Because viruses are adsorbed to solids, their presence in sludge is often increased. Payment et al. (1986) reported a mean reduction of 98 % after activated sludge treatment.

One of the objectives of this study was to establish the reduction of enteroviruses using anaerobic mesophilic treatment of sludge. These results were to be compared to the occurrence of adenoviruses in the same samples; however, adenoviruses were not detected frequently enough to make these comparisons.

Comparison of CaCo-2 and BGM Cell Lines for Enteric Viruses

The use of the CaCo-2 cell line for the improved detection of enteroviruses (Table 5.5) and enteric viruses (Table 5.6) in wastewater samples is described. The data demonstrates that CaCo-2 cells are more sensitive than BGM cells (Tables 5.4, 5.5 and 5.6). This is supported by Pinto et al. (1994) who showed consistently higher titers of laboratory strains of poliovirus 1, coxsackievirus A24, enterovirus 70, reovirus 3, human rotavirus, adenovirus 5, 40 and 41, and astrovirus 1 grown in this type of cells. CaCo-2 cells also supported the replication of hepatitis A virus (HAV) (strain HM175) but not as efficiently as in FRhK-4 cells (Pinto et al., 1994).

Statistical analysis of the data indicated that the higher MPN values in CaCo-2 cells were significant when only wastewater effluent samples were used in the Wilcoxon rank rank sum test (Table 5.6) In mesophilic treated sludge there was no statistical significance. In all positive samples PCR identified that CPE resulted from enterovirus which suggests that BGM cells (Table 5.5) (specific for enteroviruses) were less sensitive than CaCo-2. This demonstrates the ability of CaCo-2 cells to be used for monitoring of enteroviruses in environmental samples. This is supported by Pinto et al. (1995) who used CaCo-2 cells to detect wild type rotaviruses, enteric adenoviruses, enteroviruses and astroviruses in fresh water samples.

Upon analyzing the MTS and WWE samples together, statistical significance is shown (Table 6.1). The analysis of these samples grouped together was performed to increase the number of data points in the statistical analysis and thus improve the validity

of the test. To further compare the cell lines; one raw sewage sludge sample was tested. Here again there was a greater MPN value in the CaCo-2 cell line than BGM (22.4 and 136.9 MPN / 4 g. dry weight respectively). Addition of raw sewage sludge sample to the statistical analysis again showed significance ($p = 0.521$; Table 6.1).

TABLE 6.1: Statistical Analysis for the Increased Sensitivity of CaCo-2 Cell Lines for the Recovery of Enteric Viruses and Enteroviruses in Wastewater Samples

Sample set under test[1]	**p value**[2]	**Statistical significance for CaCo-2 increased sensitivity**
RSS, MTS & WWE	0.0521	yes
MTS only	0.1823	no
WWE only	0.0212	yes
MTS & WWE	0.0287	yes

[1]RSS = raw sewage sludge, MTS = mesophilic treated sludge, WWE = secondary treated wastewater effluent.

[2]p values less than 0.05 are considered significant.

Multiplex Polymerase Chain Reaction (M-PCR) in Seeded Sewage Sludge

The amplification product for each primer set are consistent with previously reported results (Allard et al., 1993; Tiemessen and Marietha, 1996). The significant size difference of each band are easily distinguished from each other for rapid visualization. In Figure 5.1 optimization of reaction concentrations revealed that buffer #1 (lane 2) containing 10mM Tris HCl, (pH 8.3) 1.5mM $MgCl_2$ and 25 mM KCl, (final concentrations) produced the brightest amplification products. Lanes 6 and 10 had the same concentrations as lane 1 but the pH was increased to 8.8 and 9.2 respectively thus reducing the yield of Ead 40 and 41 amplification product (152 bp). PCR sensitivity revealed that with the addition of only one virus type to the M-PCR reaction, the sensitivity was 10^0 (Table 5.7) In mixed viral suspensions the sensitivity was only 10^2. This could result from the competition of primers for taq polymerase or $MgCl_2$ and the formation of primer dimers (non-specific binding) all of which can decrease sensitivity (Sambrook et al., 1982)

In pure culture the sensitivity of M-PCR was as low as 10^2 $TCID_{50}$ (Table 5.7). However when single virus types were added the sensitivity was 10^0. For the seeded sewage sludge reconcentrate M-PCR was unable to simultaneously amplify the 300 bp segment of the hexon gene in Ad-2 and the 152 bp segment of the long tail fiber gene in Ead 41. Inhibition of the taq polymerase by humic acids and/or metals in the reconcentrate can result in the decrease or elimination of amplification. Schwab et al. (1995) demonstrated that detection of poliovirus in seeded beef extract mock eluates was

50 %. The authors suggested that in samples supplemented with humic acid, decreased amplicon band intensity may result from PCR interference or less efficient virus recovery.

In this study, approximately 50 % of the time the M-PCR was able to amplify the 300 bp amplicon of Ad-2 in reconcentrate seeded with Ad-2 and Ead-41 when using guanidine thiocynate/phenol (GPT) for viral DNA extraction. This method had a sensitivity of 10^3 $TCID_{50}$ in mesophilic treated sewage sludge reconcentrate. The improved DNA extraction using phenol is supported by Allard et al. (1990) who compared phenol-chloroform extraction, NaOH extraction and direct heat denaturing for adenovirus PCR on stool samples using the Hex AA1885 and 1913 primers. They determined that phenol-chloroform worked best for DNA extraction and reported a PCR sensitivity of 10^2 virus particles.

CHAPTER 7 CONCLUSIONS

1. Infectious adenoviruses were found in sewage sludge after anaerobic mesophilic treatment; however, no seasonal occurrence was observed.
2. Multiplex polymerase chain reaction for enteric adenovirus worked in cell culture stocks but was unable to simultaneously detect both adenovirus and specifically adenovirus 40 and 41 in seeded sewage sludge reconcentrates.
3. The CaCo-2 cell line was significantly more sensitive than the BGM cell line for the detection of enteric viruses in raw and treated sewage as well as disinfected secondary wastewater effluent and suggest to be more sensitive than BGM cells for enteroviruses.

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