SOMATIC COLIPHAGE FAMILIES AS POTENTIAL INDICATORS OF ENTERIC VIRUSES IN WATER AND METHODS FOR THEIR DETECTION

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ABSTRACT

HEE SUK LEE: Somatic coliphage families as potential indicators of enteric viruses in water and methods for their detection
(Under the direction of Mark D. Sobsey)

The potential use of specific somatic coliphage taxonomic groups as viral indicators on the basis of their persistence and prevalence in water was investigated. Representative type strains of the 4 major somatic coliphage taxonomic groups were seeded into environmental water and their survival was measured at temperatures of 23-25 and 4°C. Based on their greater persistence over time, the *Myoviridae* (type strain T4), the *Microviridae* (type strain PhiX174), and the *Siphoviridae* (type strain Lambda) were the preferred candidate somatic coliphages as candidate fecal indicator viruses in water. Also, a conventional, group-specific PCR method was developed to identify each of the 4 major taxonomic groups of somatic coliphages and used to classify individual somatic coliphage isolates from primary human sewage effluent to further document those detected and to describe their behavior in environmental waters. Over time, the taxonomic makeup of the somatic coliphage population in sewage changes, with the *Microviridae* family becoming the most prevalent taxonomic group in the sewage population after several weeks. Based on their persistence and prevalence in environmental waters, phages belonging to the *Microviridae* family could serve as indicators for sewage contamination and perhaps human enteric viruses in water.

Rapid detection methods for reliable viral indicators that predict viral contamination in water are essential for timely protection of public health. Individual somatic coliphage

families that are relatively persistent and abundant in environmental waters are possible reliable viral indicators. Rapid detection of the *Microviridae* family of somatic coliphages by real-time PCR method was developed and successfully applied to environmental water samples: primary sewage effluent of two different geographic regions, seawater, and groundwater. Also, as an antibody-based rapid detection method, CLAT (Culture, Latex Agglutination, and Typing), for the *Siphoviridae* family, N4-type viruses of the *Podoviridae* family, and T4-type viruses of *Myoviridae*, was developed and successfully applied to somatic coliphage isolates, although there is a need for improvement in method sensitivity and specificity. Developing new and rapid nucleic-based detection and antibody-based somatic coliphage detection and characterization methods will assist in future studies to evaluate individual somatic coliphage families as sewage and viral indicators for water quality assessment.

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CHAPTER 1 Introduction

Background

Contamination of water is a continued threat to public health worldwide. Sources of water contamination include improperly functioning wastewater treatment plants, septic tank leakage, latrine and landfill leakage, storm and urban runoff, zoonotic animal waste and other fecal sources. Due to greater numbers and diversity of water contamination sources, it is difficult to identify and quantify all of the potential microbial agents in contaminated water. For decades, microbial indicators for estimating fecal contamination in water have been used for defining levels of microbiological quality in environmental waters. Detection of microbial indicators serves as a simple diagnostic tool to predict microbiological water quality with respect to pathogen presence and densities and human health risks. Currently, total coliforms, fecal coliforms, *enterococcus* spp., and *E. coli* are used as microbial indicators for predicting water pollution. Also, these bacterial indicators have shown good correlation with illness risks in people who contact and ingest water (Cabelli, 1983; Dufour, 1984).

Although enteric bacterial indicators have been used as tools to estimate the microbiological quality of environmental waters, many waterborne pathogens that cause illness and diseases in humans are enteric viruses. Bacterial indicators may be inadequate or unreliable indicators for estimating enteric virus presence in contaminated water. Compared to bacterial indicators, enteric viruses have shown higher survival rates during wastewater

and drinking water treatment and greater persistence in environmental waters (Contreras-Coll *et al*., 2002; Duran *et al*., 2002; Harwood *et al*., 2005; Moce-Llivina *et al*., 2005; Jofre *et al*., 1995). Therefore, there are possibilities that bacterial indicators alone could underestimate microbiological contamination of water and the associated human health risks. Due to higher persistence and resistance of waterborne enteric viruses as compared to bacterial indicators, water which is defined as safe based on bacterial indicator measurement may still cause disease or illness to consumers due to the enteric viruses still present (Dore *et al*., 2000; 2003; Formiga-Cruz *et al*., 2003). Therefore, bacterial indicators alone do not provide a complete assessment of microbiological water quality. Adding at least one viral indicator for estimating viral presence and behavior in environmental water potentially can give better assessment of virological water quality and provide confidence of virological water safety.

There are several candidate viral indicators to predict virus contamination in environmental waters. Several studies indicate that bacteriophages could serve as viral indicator for estimating enteric viruses in water (Havelaar, 1993; Kott, 1981). Three groups of bacteriophages have been proposed as potential viral indicators for estimating sewage contamination and human enteric viruses in water based on their similarities to human enteric viruses in morphology, nucleic acid composition, sources and occurrence in contaminated water: F-specific RNA coliphage, phages of *Bacteroides fragilis*, and somatic coliphages. Havelaar *et al*. (1993) showed significant correlation of F-specific RNA bacteriophages and enteric viruses in river water and lake water. Even though, F-specific RNA coliphages are a relatively abundant and homogeneous group, their survival in natural water is less than some enteric viruses (Jofre, 2002). Bacteriophages infecting *Bacteroides fragilis*, an anaerobic bacterium which is found in the human intestinal tract, showed correlations in numbers with

enteroviruses and rotaviruses in sewage related sediment (Jofre *et al.*, 1989). However, occurrence of phages of *Bacteroides fragilis* in human feces is relatively low (Gantzer *et al.*, 2002). Stetler (1984) investigated the occurrence of enteroviruses and somatic coliphages in river water and found that enterovirus showed better correlation with coliphages than they did with bacterial indicators of total coliforms, fecal coliforms, fecal streptococcus or plate count bacteria.

Somatic coliphages are DNA viruses of *E. coli* and perhaps related bacteria. They infect via a lipopolysaccharide on the *E. coli* cell outer surface and have been examined as fecal indicators in studies of sewage and drinking water treatment plant source water. There are four taxonomic families in the somatic coliphage group: *Podoviridae, Microviridae, Myoviridae,* and *Siphoviridae* (ITCV 2007). Somatic coliphages can be distinguished in standard plaque assays by their ability to infect the cell wall of F-minus *E. coli* hosts such as strains C and CN-13, which lack the ability to form pili. Somatic coliphages are routinely found in human sewage (Mocé-Llivina *et al.*, 2005) and are more prevalent than F+ RNA coliphages in marine water and warm waters (Lovelace *et al.*, 2005). However, somatic coliphages are a heterogeneous group because they belong to different taxonomic groups with different morphologies and other characteristics. Previous studies investigating somatic coliphages as a viral indicator have treated somatic coliphages as one whole group even though they are taxonomically, genetically, morphologically, and otherwise diverse and heterogeneous. This diversity might account for the lack of the correlation of somatic coliphage and enteric virus occurrence in environmental waters. Therefore, there is need to investigate individual somatic coliphage families as promising viral indicators of sewage contamination and enteric viruses in water.

Genetic characterization of the occurrence and distribution of somatic coliphage families in fecal sources, recreational water, and shellfish has not been done to date. Such genetic characterization is needed to determine the occurrence, fate and potential indicator value of the different families of somatic coliphages. Development of new tools to identify and characterize each somatic coliphages family would make it possible to characterize individual somatic coliphage families and subgroups rather than consider the whole heterogeneous group. Furthermore, identifying the most persistent and abundant taxonomic family(s) of somatic coliphage could give better understanding of their sources, occurrence and distribution in environmental waters. Therefore, the research priority identified for this study is the development and evaluation of new screening technologies to detect, identify and characterize different somatic coliphage families as microbial indicators of fecal contamination sources and their associated viral pathogens.

Individual families, subgroups and strains of somatic coliphages may be potential microbial indicators of fecal contamination sources. Previous studies suggest that the *Myoviridae* family of somatic coliphages is the predominant type in human sewage, and the *Siphoviridae* family is most common in surface waters (Ackermann and Nguygen 1983; Pedroso and Martins 1995; Muniesa *et al.,* 1999). Other studies have found poor correlation between somatic coliphages and bather disease risks in recreational seawaters but good predictability between somatic coliphage and bather disease risks in fresh recreational water (Wiedenmann *et al*., 2006). This inconsistent correlation with gastrointestinal illness risk in bathers may be a consequence of the use of current coliphage detection methods that have a low specificity for sewage-associated somatic coliphages by detecting heterogeneous groups of somatic coliphages as a whole (Colford *et al.,* 2007; Widenmann *et al*., 2006). Developing

new methods with greater specificity for sewage-associated somatic coliphages by identifying each somatic coliphage family would improve future studies on their usefulness as indicators of water quality and human health risks. The basis and approach of this work is similar in nature to previous work identifying *E. coli* as a more feces-specific indicator bacteria than total coliforms (Dockins and McFeters, 1978) and a better predictor of gastrointestinal illness risks, as from sewage contaminated recreational waters (Dufour, 1984).

Standard culture-based bacterial indictor assays take 1-4 days, resulting in water quality information and decisions that lag days behind actual contamination events and human exposures. Even though there are candidate reliable fecal indicators to predict fecal contamination or presence of enteric viruses in environmental waters, rapid detection of those indicators is essential to provide timely protection of public health as safe water to consumers and users. The timely regulation of contaminated water and proper legislation to achieve and protect safe water are strongly dependent on the detection of indicator microorganism in a timely manner at least before the water is used by consumers. In previous studies, simple, rapid and field-ready detection methods have developed and applied for the detection of F-specific RNA coliphages in field samples of water (Love *et al.*, 2007). Latex agglutination assay of antigen and antibody reactions was originally developed for clinical uses (Hughes *et al.*, 1984). Recently, a coliphage latex agglutination test (CLAT) was successfully developed and applied to environmental field samples for rapidly detecting and identifying (serotyping) F-specific coliphages (Love *et al.*, 2007). It is hypothesized that the successful application of CLAT assay as a rapid detection method for somatic coliphage detection and typing is possible if antisera against specific somatic coliphage families and

subgroups show sufficient sensitivity and specificity, analogous to that achieved for F+ coliphages. In addition to an antibody-based rapid detection method such as the CLAT, real-time PCR methods based on nucleic acid detection using specific primers and probes is another promising rapid detection method worthy of consideration that has already been applied to the detection and characterization of F+ RNA coliphages.

In summary, there is a need for reliable viral indicators of sewage contamination and human enteric viruses and rapid, simple methods to detect them in water monitoring programs for source water, water and wastewater treatment and recreational and shellfish water management. Somatic coliphages and their individual families, subgroups and strains are candidates to fill this need. To evaluate individual somatic coliphage families as candidate viral indicators of enteric viruses, it is necessary to 1) identify the most persistent and abundant somatic coliphage families, and to 2) develop rapid methods that specifically detect these candidate viral indicators in water.

Objectives

Somatic coliphages as individual families, subgroups and strains will be evaluated as potential indicators for estimating the presence of human enteric viruses in water on the basis of their persistence and abundance. To achieve this objective, there are two approaches: one is by survival tests of prototype positive strains in model and environmental waters, and the other is by determining the most persistent and abundant somatic coliphage family(s) in primary sewage effluent. Rapid methods for the detection of somatic coliphage families will be developed and evaluated by rapid molecular and immunological detection methods for them. Also, newly developed rapid detection methods will be applied to different water

samples representing different regions or sample matrices. Overall, to evaluate an individual somatic coliphage family as a candidate viral indicator of sewage contamination and enteric viruses, there is a need to 1) identify the most persistent and abundant somatic coliphage family(s) and 2) develop and evaluate methods that rapidly, efficiently and specifically detect these candidate viral indicators in water. To identify candidate somatic coliphage families to serve as indicators of human enteric viruses in water, and optimize methods for their detection, this work was undertaken with the following specific aims:

- **Specific Aim 1:** Determine if somatic coliphages are potential indicators for the presence of sewage and human enteric viruses in surface water, based on identifying the most persistent and prevalent taxonomic group(s) in sewage and fecally contaminated environmental waters. To achieve this aim, at least one representative prototype strains of each family (*Microviridae, Myoviridae, Siphoviridae, Podoviridae*) were spiked to reagent grade water and surface water and held at two different temperatures, 4 °C and 25°C, for 90 days. Aliquots of each sample were taken periodically to determine remaining virus concentrations and thereby measure the inactivation rate of each somatic coliphage strain on a weekly basis. After 90 days, the most persistent family could be identified by measuring the relative survival rate of each strain.

- **Specific Aim 2**: Identify the most persistent and abundant somatic coliphage families in primary sewage effluent of wastewater treatment plants by survival tests at 4°C and 25°C. At different incubation times, presumptive somatic coliphage isolates were

collected from virus infectivity assays and these were archived to further determine and characterize their families. To determine the family of somatic coliphage isolates, group-specific conventional PCR of four families of somatic coliphages (*Microviridae, Myoviridae, Siphoviridae, Podoviridae*) was developed and optimized by applying the new methods to available positive strains of each subgroup in each family. After optimization of group-specific conventional PCR, the methods were applied to somatic coliphage isolates to identify their families. From group-specific PCR analysis of the isolates obtained over the time course of survival tests, families and subgroups of somatic coliphage families could be identified, and the most abundant and persistent families determined.

- **Specific Aim 3:** Develop and evaluate molecular and immunoassay analytical methods for rapid detection and typing of somatic coliphage groups (families and subgroups) potentially indicative of sewage contamination and the presence of human enteric viruses of fecal origin in water. Real-time PCR methods were developed and optimized for the *Microviridae* family of somatic coliphages because of their higher persistence and abundance in tested sewage and fecally contaminated water compared to other families. Antibody based rapid detection methods were also applied to somatic coliphage families by developing polyclonal antisera of representative somatic coliphage strains in each family (*Microviridae*, *Myoviridae*, *Siphoviridae*, *Podoviridae*). After confirming sufficient sensitivity and specificity of each antisera against somatic coliphage strains or families, rapid coliphage CLAT (Culture, Latex Agglutination, and Typing) (Love *et al.*, 2007) assay will be applied to somatic

coliphage isolates for screening their families or strains as indicators of sewage and fecally contaminated water.

- **Specific Aim 4:** Apply the developed rapid and specific detection methods for somatic coliphage families by real-time PCR and antibody-based CLAT assay to different water types, including primary sewage effluent, seawater and groundwater samples. The developed rapid detection methods of somatic coliphage families by real-time PCR and antibody-based CLAT assay should be applicable to different water types. Regionally different primary sewage effluents will be tested by group-specific conventional PCR and real-time PCR for the *Microviridae* family of somatic coliphage because they were found to be both abundant and persistent in sewage. To determine the most persistent and abundant somatic coliphage families in samples, survival tests were performed. Additionally, a method for rapid real-time PCR detection of the *Microviridae* family was developed and applied to seawater and groundwater samples.

Literature review

Introduction and background

To consider somatic coliphage families as a potential viral indicator in water, several aspects of ideal indicator definitions were investigated to explore the individual somatic coliphage families as candidate fecal indicators of sewage contamination and of enteric viruses. Taxonomically, somatic coliphages were composed of four families among 13 bacteriophage families, and they showed a greater diversity in morphology and genetics.

Therefore, the classification of somatic coliphages families and their properties is reviewed here.

Ideal indicator microorganism must meet several criteria to be considered useful for detecting fecal pathogens in environmental waters. Indicator microorganism should persist and survive greater than or equal to pathogens. Therefore, the survival of potential indicator of enteric viruses should be longer than the survival of enteric viruses. Survival of somatic coliphage will be discussed on the basis of their use as potential indicators. An ideal indicator should be present when the pathogen is present, so the occurrence of somatic coliphages as candidate indicators of enteric viruses in fecally contaminated water was considered on the basis of their ability to predict fecal contamination. There have been many studies on the examination and identification of candidate fecal indictor microorganism of enteric viruses for decades, and coliphages were found to be good viral indicators in numerous studies. However, some studies presented contradictory findings. Here, the weaknesses and strengths of individual somatic coliphages as a potential viral indicator will be discussed. In addition, an ideal indicator should be detected in a short time by simple methods. So, the approach of rapid detection of somatic coliphage was considered in this review.

Classification of bacteriophages and somatic coliphages: The International Committee on the Taxonomy of Viruses (ICTV) classifies bacteriophages into 13 families. These virions have binary (tailed), cubic, or helical symmetry, or pleomorphic shape. Families are mainly defined by the properties of the nucleic acids and phage morphology. Phages can contain double-stranded DNA, single-stranded DNA, double-stranded RNA, or single-stranded RNA. Tailed phages with binary symmetry, are classified in three families,

Myoviridae (Tail contractile), *Siphoviridae* (Tail long, noncontractile), and *Podoviridae* (Tail short). The *Microviridae*, one of the families having cubic symmetry, contains single-stranded DNA having circular morphology. These groups are highly diverse (Achermann, 2006). Four of the 13 phage families, *Myoviridae*, *Siphoviridae*, *Podoviridae*, and *Microviridae*, are grouped as somatic colipahges.

Somatic coliphages use a lipopolysaccharide (LPS) on the *E. coli* cell outer surface as a recognition factor to initiate infection. They infect mostly *E. coli* but some of them can infect other *Enterobacteriaceae.* The *Microviridae* group infects a diverse range of hosts such as *Enterobacteria, Bdellovibrio, Chlamydia,* and *Siroplasma*. For the *Myoviridae* group, principal hosts are *Enterobacteria, Bacillus,* and *Halobacterium*. For the *Siphoviridae* group, *Enterobacteria, Mycobacterium,* and *Lactococcus* are the major host groups. For the *Podoviridae* group, *Enterobacteria* and *Bacillus* are the main host groups.

The first step in the cycle of infection is recognition by a phage attachment site on the bacterial surface. This receptor may be part of a protein, a lipopolysaccharide, a teichoic acid, the peptidoglycan, or an exopolysaccharide. The specificity of the receptor depends on both its composition and spatial configuration. Initial attachment following recognition is reversible, allowing the phage to (re-)position itself. This is followed by irreversible steps involving triggering of conformational changes in the particle (Coetzee, 1984).

Coliphage replication processes and conditions in the environment: F-specific coliphages are single-stranded RNA viruses that infect the cell via the F-pili. In general, F specific phage infection does not occur at temperatures below 30°C because the host *E. coli* does not produce F pili at low temperatures. However, if F+ coliphages produced at

temperatures at or above 30°C are present with phages at temperatures below 30°C, adsorption, infection and replication can occur, depending on the temperature and other environmental conditions. Somatic coliphages infect a lipopolysaccharide on the *E. coli* cell outer surface, so the factors governing their potential for their extra-intestinal replication outside of a mammalian host and in the environment are different. While there is some restriction on the environment temperature conditions for their attachment to and infection of host cells for replication, other factors may also strongly influence the potential for such infection and replication processes.

The potential for replication of the somatic coliphages in the environment has been considered as a weakness of somatic coliphages as suitable viral indicators in water. This replication could be affected by several factors such as the densities of host bacteria and phages, the physiological condition of the host bacteria, the dissolved and suspended solids in the water, the temperature, the other bacteria present in water and other factors. Muniesa *et al.* (2004) studied the factors affecting somatic phage replication using *E. coli* strain WG5. They concluded that there is little chance for replication of somatic coliphages in environmental waters, though it cannot be ruled out completely. The threshold densities of host bacteria and phages which they used are greater than the highest densities of somatic coliphages and host bacteria reported in most human and animal raw wastewaters. Therefore, they reported that there are few natural environments in which the densities of non-replicating host bacteria and their physiological status support somatic coliphage replication. They also concluded that the ratio of phages to bacteria will not be affected by replication in water, and consequently, the likelihood of somatic coliphage replication is very low outside of the animal gut. Woody *et al*. (1995), examined factors affecting F-RNA

coliphage replication, and found that phage infection of the host is decreased at lower temperature because very few host cells have F pili. Even though F-RNA coliphages infect host cells at 25°C, their findings indicated that F-RNA coliphage replication would be limited. Therefore, there is no clear evidence that there is a higher likelihood of somatic coliphage compared to male specific coliphage replication in aquatic environmental systems.

Survival in the environment and association with fecal contamination: Enteric virus particles persist relatively long in environmental waters, though the viral particles may not remain infectious. There have been several survival tests of male specific coliphages compared to enteric viruses. Chung *et al.* (1993) reported that F+ coliphage showed greater inactivation than enteric viruses at 25°C in seawater, and suggested that F+ coliphage may not be a suitable indicator of certain human enteric viruses in the warmer summer season. However, Lees and colleagues found that F+ coliphages were good indicators of the presence of noroviruses in shellfish and risks of gastrointestinal illness from such shellfish in the relatively cold waters of the United Kingdom (Dore *et al*., 2000). As there are geographical differences and seasonal variations in their inactivation rate compared to enteric viruses, F+ coliphage may not always be an appropriate viral indictor in seawater or shellfish.

There are relatively little data on survival of somatic coliphages in environmental waters compared to male-specific RNA phages. Some research on T3 coliphage survival in aerosols was conducted by Warren *et al.* (1969), who found that this coliphage is relatively stable in aerosols, declining by only 2 $\log_{10}$ in 72 days at 4°C. These results suggest that somatic coliphages may be stable for long periods in the environment. Callahan *et al.* (1995) compared survival of hepatitis a virus, poliovirus and indicator viruses in seawater; survival

was greater for somatic *Salmonella* bacteriophages (SS phages) than the other viruses, including FRNA phages, evaluated in the study. Also, Chung *et al*. (1995) found that *Bacteroides fragilis* phages survived longer than or comparable to enteric viruses in seawater and seawater-sediment mixtures. More recently, Rose and colleague (2006) found that *Bacteroides fragilis* phages persisted longer compared to coliphages and showed little variation between the temperatures in the seawater.

In the work of Havelaar *et al*. (1990), somatic coliphages were detected in most fecal samples, often in relatively high concentrations. They noted that there are no consistent relationships between counts of somatic and F-RNA coliphages that were detected in any of the animal species examined. According to the work of Osawa *et al.* (1981), the RNA phages constitute only a minor fraction of the total coliphages in the feces of human and animals. Dhillon *et al.* (1976) studied the distribution of coliphages in mammalian feces with characterization for selected isolates. Based on host strains and neutralization with antiserum, the phages were identified as T1, PhiXI74 or S13-related. T1-related phages were recovered from both cow and pig feces. The S13-related phages were more likely to be found in porcine fecal matter while PhiX174-related phages were found in fecal samples of bovine origin. When isolates from rivers, human and animal fecal samples were classified into families based on their morphology, phages were found to be largely from the *Siphoviridae* and *Myoviridae* subgroups of somatic coliphages.

Somatic coliphages as a fecal indicator of enteric viruses: The potential for somatic coliphages to serve as viral indicators has been studied by a number of researchers. Many of these studies focused on male-specific coliphages as viral indictors, due to morphological

similarity to enteric viruses. There are advantages and disadvantages to the use of male-specific coliphages as viral indicators in environmental waters, and there is no regulatory consensus on coliphages as viral indicators up to now. Somatic coliphages can be evaluated across several dimensions of suitability as viral indicators. Some of these are listed below (Phage Ecology, 1984; The Bacteriophages, 2006)

- Somatic coliphages are found abundantly in wastewater and waters of impaired quality which are used for source water in drinking water treatment systems.
- The populations of somatic coliphages in source waters exceed those of enteric viruses
- Somatic coliphages show relatively high persistence through water treatment plant processes compared to typical bacterial indicators. Somatic coliphages may behave similarly to human enteric viruses during water treatment process, including in their responses to disinfection.
- Somatic coliphages can be detected by relatively rapid, simple, affordable, and robust culture methods compared to enteric viruses during the processes of water treatment and in other water and environmental media.
- Somatic coliphages are more resistant to inactivation by adverse environments and disinfection processes than enteroviruses, and they are reduced less effectively than are conventional indicator bacteria by water treatment processes.

Stetler *et al.* (1984) found that coliphages showed better correlation with enteric virus presence than did total coliforms, fecal coliforms, fecal *streptococci,* or standard plate count organisms. Payment *et al.* (1993) reported that only somatic coliphages infecting the host *E. coli* CN13 were found to be an explanatory variable for the presence of human enteric

viruses in settled water by multiple regression analysis. Also, they suggest that coliphages could be indicators of drinking water treatment efficiency. They also reported that there does not seem to be any advantage in using male-specific coliphages over somatic coliphages as viral indicators; both have similar behavior during treatment, but somatic coliphages are easier to detect.

However, Vaughn and Metcalf (1975) suggested that coliphages are not suitable indicators of enteric viruses according their research. They reported (a) coliphages were consistently present in raw sewage samples that yielded inconsistent enterovirus isolations, (b) treated sewage effluents were coliphage positive but enterovirus negative, and (c) many (63%) enteroviruses isolation occurred without phage isolation. In addition, Gino *et al.* (2007) reported that *E. coli* F specific host survived better than other host strains in sewage, and that F specific phages were more frequently isolated from sewage at higher numbers while somatic coliphages were absent. Also, Colford *et al.* (2007) reported that somatic coliphages were not effective indicators for assessing risks of GI disease from exposure to marine bathing water. In addition, they suggested an alternative fecal indicator, perhaps F+ coliphages, for monitoring beach water having major non-point fecal contaminations. In contrast, Widenmann et al. (2006) reported that somatic coliophage and a number of enteric bacterial indicators were good predictors of risk of gastrointestinal illness from fecally contaminated fresh recreational water.

It is noteworthy that the *E. coli* hosts, assay procedures, sample processing procedures and other variables in coliphage detection used in different studies could cause differences in coliphage results and predictive abilities. In addition, many sewage systems often contain both somatic and F specific coliphages at similar levels. Sometimes F specific coliphages

may outnumber the somatic coliphages in some environments and other times the reverse is reported. Overall, there is little information on the ecology, sources and concentrations of somatic coliphages in sewage or various environmental waters. Furthermore somatic coliphages are a relatively diverse and broad taxonomic group. Therefore, more research is needed to evaluate somatic coliphages as suitable viral indicators in water treatment process, as indicators of sewage contamination and for the presence and human health risks of enteric viruses in fecally contaminated waters.

Potential use of somatic coliphages as an indicator microbe for source tracking: There are numerous studies on the possibilities of male specific coliphages as microbial source tracking tools. Stewart *et al.* (2006) concluded that F+RNA coliphage analysis can be used for source identification of fecal contamination in surface waters. In this study, they suggested that F+ coliphage typing can provide useful, but not absolute, information to distinguish human from animal sources of fecal pollution for water quality assessments. If F+RNA coliphages have the potential to be used as a possible microbial source tracking tool, somatic coliphages also have the possibility to be used as a potential microbial source tracking microorganism. The extent to which it is possible for somatic coliphages to be used as human or animal fecal contamination indicators depends on the extent to which specific ones (families and subgroups) can be shown to be associated only with fecal contamination from particular sources such as humans or certain animals. If there are one or two fecal specific subgroups of somatic coliphage from particular sources, such as humans or specific kinds of animals that impact in environmental waters, such families or subgroups might be

suitable species-specific microbial source tracking tools that are comparable to or even more specific than geno-groups of F+ coliphages.

Somatic coliphages may also be useful as indicators of water treatment process efficacy. Some studies showed that water treatment by coagulation-flocculation processes with alum removed 98% and 99% of T4 and MS-2 respectively. From this result, both phages can be removed in coagulation-flocculation process to levels comparable to those for human enteric viruses (Sobsey *et al*., 1995). Also, in the presence of sewage effluent, T2 adsorption to activated carbon varied between 29 and 75% in batch experiments and 7 and 32% in continuous flow studies. Therefore, activated carbon is generally a poor adsorbent of phage and human enteric viruses due to the competitive adsorption of organic matter onto activated carbon (Bitton, 1984). Bitton (1984) concluded that rapid sand filtration and activated carbon processes do not provide stable and effective removal of phages, and that phage removal rates are dependent on the water quality.

In some previous disinfection studies, RNA phage f2 and DNA phage T2 inactivation was compared with several disinfectant conditions. Both phages were more sensitive to disinfection than was poliovirus, but more resistant than coliform bacteria. However, other studies indicate that F+ coliphages like MS2 are inactivated comparably to human enteric viruses like poliovirus by free chlorine, chloramines, chlorine dioxide and ozone. Far less is known about the response of somatic coliphages to these disinfectants. The results for F+ coliphages suggest that these phages may be better indicators of the response of viruses to disinfection processes than current bacterial indicators. There is evidence that some subgroups of somatic coliphages differ in their response to UV disinfection. Compared to representative human enteric viruses some somatic coliphages are more resistant, others are

less resistant and yet others are as resistant to UV disinfection (Shin *et al*., 2005). Some somatic coliphages may be similar to the enteric adenoviruses 40 and 41, with high resistance to UV disinfection. Because Adenoviruses and certain somatic coliphages such as the *Tectiviridae* contain double-stranded DNA viruses and have similar morphology, their UV resistance also may be similar. Stetler *et al*. (1984) showed that enteroviruses were better correlated with coliphage than with the traditional bacterial indicators in a study of water treatment plants in Michigan. However, there are still only limited data available to propose coliphages as ideal or acceptable indicators of enteric viruses in water treatment processes and systems.

In a previous review of water treatment efficiency of virus removal and inactivation of somatic coliphages and human enteric viruses, somatic coliphages were evaluated as a general and therefore heterogeneous group in their response to water treatment process such as physical-chemical treatment and disinfection (Payment *et al*., 1993). These comparisons are difficult to interpret because of the heterogeneity of somatic coliphages in terms of families, subgroups and their differing properties and characteristics. Because available somatic coliphage data are limited and do not adequately consider the individual families, subgroups and strains, different results may be observed for their responses to water treatment process and in comparisons to human enteric viruses.

Rapid detection methods for somatic coliphages: There have been many studies to develop rapid detection methods of fecal bacterial indicators using substrate/enzyme, illuminance, nucleic acid approaches, and antibody based methods. This is because the timely management and regulation of microbiological water quality in source recreational,

and shellfish waters are important to protect public health. Rapid detection is as important as identifying the right indicator microorganism to estimate fecal contamination and possible pathogen presence in water. There have been many studies to find reliable viral indicators to predict the occurrence of human enteric viruses in water. Some studies also focused on developing rapid detection methods for candidate viral indicators, such as those described by Love *et al*. (2007), in which rapid detection of F+ coliphages was based on methods previously developed for the rapid detection of bacterial indicators (Noble *et al*., 2004). Two approaches are promising for rapid detection: nucleic acid-based and antibody-based methods, each of which has advantages and disadvantages in terms of sensitivity, specificity, cost, time, and accuracy.

Real-time PCR is a nucleic acid based detection method that is rapid and sensitive for detection of target microorganisms. There are some studies targeting coliphages for rapid detection by real-time PCR (Kirs *et al*., 2006; Ogorzaly *et al*, 2006), and they focused on detection of F-specific coliphages. Even though real-time PCR is a promising method for rapid detection of indicator microorganisms of interest, the disadvantages of this method include difficulty in distinguishing the infectivity of target microorganisms, and difficulty in application to field situations where simplicity, portability and independence from sophisticated laboratory infrastructure, equipment and electricity are desirable attributes of the methods. However, if real-time PCR is applied to coliphage detection after culture enrichment of coliphage in samples, the infectivity issue is largely addressed by the inclusion of this initial culture step.

Finding associations between fecal sources and specific somatic coliphage taxonomic families has the potential to improve usefulness of this real-time PCR method, by applying

to more than one target somatic coliphage group, each of which may have different or predominant sources. A previous study by Kirs and Smith developed a multiplex real-time RT-PCR method for F+ RNA coliphages. However, they used just seven unique full length nucleotide sequences (Kirs *et al*., 2006), which is less than the ideal or preferred number to be representative for generating robust primers and probes. Recently, Friedman et al. (2009) reported full-length nucleotide sequences for 30 F+ RNA coliphages, which provides more comprehensive data from which to develop robust primers and probes for real-time PCR.

In one candidate somatic coliphage family, the *Microviridae*, a total of 43 full length sequences are available through the National Center for Biotechnology Information (NCBI), which greatly increases ability to develop robust primers and probes of high sensitivity and specificity for *Microviridae* amplification. In order to provide better real-time PCR detection, intercalating dyes such as SYBR Green can be used to determine melting curves to better characterize probe binding temperatures for optimization of the process. If this is successful, new real-time PCR methods can provide rapid (<4 hr) results for detection, quantification, and source tracking of feces-specific, abundant, and persistent somatic coliphages.

Summary

The potential for individual families or subgroups of somatic coliphage to be a useful viral indicator of fecal contamination in environmental water was not taken into consideration in previous studies. Only the broad heterogeneous group of somatic coliphage was considered as a candidate viral indicator. The greater diversity of the somatic coliphages group in terms of genetics, morphology, removal by treatment processes and resistance to disinfection and environmental stressors may contribute to the lack of specificity of this

broad group. It is possible that the persistence, response to treatment and prevalence of individual families of somatic coliphages might be different, and one or two of families could have prevalence and survival properties to be useful as reliable viral indicators of sewage contamination and enteric viruses in water. The extent to which the properties of an ideal indicator are met by somatic coliphages as potential sewage and viral indicators, based on prevalence and persistence is considered in the research chapters that follow.

The limitation of detection methods to distinguish between each somatic coliphage family restricts the study of individual families as indicators. To overcome this limitation of the lack of specific somatic coliphage family detection, the approach of molecular typing of somatic coliphage families was explored on the basis of the individual families being possibly reliable indicators. Additionally, the approach of exploring and evaluating rapid detection method for individual somatic coliphage families was investigated. These approaches were pursued for the purpose of determining if individual somatic coliphage families had useful properties as a potential indicator of sewage contamination and enteric virus and for understanding the ecology and distribution of somatic coliphages in environmental waters.

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CHAPTER 2 Survival of prototype strains of somatic coliphage families in environmental waters

Abstract

The potential use of specific somatic coliphage taxonomic groups as viral indicators based on their persistence and prevalence in water was investigated. Representative type strains of the 4 major somatic coliphage taxonomic groups were seeded into reagent water and an ambient surface water source of drinking water and the survival of the added phages was measured over 90 days at temperatures of 23-25 and 4°C. Based on their greater persistence in water over time, the *Myoviridae* (type strain T4), the *Microviridae* (type strain PhiX174), and the *Siphoviridae* (type strain Lambda) were the preferred candidate somatic coliphages as fecal indicator viruses in water.

Introduction

Fecal indicator microorganisms currently used for water quality monitoring are bacteria, such as total coliforms, fecal (thermotolerant) coliforms, *Enterococcus* spp. and *E. coli*. However, many waterborne pathogens are enteric viruses, and bacterial indicators may be inadequate or unreliable indicators of their presence, persistence and concentrations in environmental waters. To ensure adequate protection against waterborne disease, there is a need for reliable viral indicators in water quality monitoring programs. Although there have been a number of previous studies to find reliable viral fecal indicators (Armon, 1993;

Havelaar *et al*., 1993; Jofre *et al* 1995), there is no clear evidence of reliability and no clear consensus as to which fecal indicators are most predictive of human enteric viruses.

Certain types of bacteriophages, specifically phages of *Escherichia coli* (Coliphages) are proposed candidate indicators of human enteric viruses in water. They are present in human and animal feces and some are small, icosahedral and non-enveloped viruses, making them structurally similar to many human enteric viruses. There are two main types of coliphages: somatic and the male-specific (F+). The somatic coliphages are DNA viruses that infect *E. coli* through attachment to specific sites on the outer cell layer, such as lipopolysaccharide. The male-specific coliphages are single-stranded RNA and DNA viruses that infect the cell via the pili appendages present on the surface of male strains of the bacterium. Male-specific coliphages have been previously investigated as viral indicators (Colford *et al.*, 2007, Dore *et al.*, 2000, Cole *et al.*, 2003, Love and Sobsey, 2007). However, their infrequent presence in human feces, their relative scarcity and their rapid die-off rates in warm waters (Love *et al*, 2007) limit their usefulness of F+ coliphages as indicator viruses. Somatic coliphages also have been proposed as fecal indicators of human enteric viruses in studies of sewage, source water for drinking water and marine waters (Moce-Llivina *et al.*, 2005; Muniesa *et al.*, 2007; Jofre, 2008), and real-time monitoring of somatic coliphages as fecal indicators has been suggested by previous investigators (Araujo *et al.*, 1997; Skraber *et al.*, 2004, Garcia-Aljaro *et al.*, 2008).

Although previous studies provide some evidence that somatic coliphages are potentially useful candidates as fecal indicator viruses, their taxonomic diversity and potential heterogeneity within a taxonomic group has not been taken into account. The somatic coliphage group encompasses four distinct virus families, each containing several

genera: *Myoviridae, Microviridae, Siphoviridae*, and *Podoviridae*. The *Microviridae* are small, single-stranded DNA viruses, and the other families are double stranded DNA viruses of varying size, morphology and biophysical properties. It is possible that the survival of these different phages in environmental waters may differ among families and genera, with some being more persistent in water than others. Due to the diversity of the somatic coliphage group, more data on the comparative persistence and prevalence of representative members of the different somatic coliphage families are needed to identify which somatic phages may serve as a reliable viral indicator in water based on their survival. In order to identify a candidate somatic coliphage family for use as an indicator for the presence of human enteric viruses in surface water, we evaluated the comparative persistence in water of representative members of each of the 4 taxonomic group(s) using established prototype strains.

Materials and Methods

Test waters Test waters used were reagent water and a natural surface water. For the reagent water, Dulbecco's phosphate buffered saline (PBS) solution was diluted 10-fold in reagent-grade water. Reagent-grade water was produced from laboratory tap water by a Dracor™ water purification system (Dracor, Durham, NC) which includes reverse osmosis and ultraviolet light treatment. Ten-fold diluted Dulbecco's PBS was used to provide salt content (total dissolved solids) in the range of fresh water. Surface water was obtained from University Lake (Temperature : 18.2 °C, Turbidity: 4.5NTU, pH:6.6, Alkalinity: 25 mg/L, Hardness: 29 mg/L, TOC: 6.80 mg/L, DOC: 6.22 mg/L, Total coliform: 1472 colonies/100mL, *E. coli* 66 colonies/100mL, Heterotrophic Plate Count: >5700 CFU/mL), an

impoundment that serves as the source water for the Orange County Water and Sewer Authority (OWASA) drinking water treatment facility serving Chapel Hill, NC.

Coliphage propagation Type strains of somatic coliphages used in this study were PhiX174 (*Microviridae*), T4 (*Myoviridae*), T7 (*Podoviridae*), and T1 and Lambda (*Siphoviridae*). Phages and their bacterial hosts were obtained from the Felix d'Hérelle Reference Center for Bacterial Viruses, University of Laval, Canada. T1, T4, and T7 were propagated in host *E. coli* B. Hosts for PhiX174 and Lambda were *E. coli* C and *E. coli* K12S Lederberg, respectively. Host strains were grown in tryptic soy broth (TSB; Difco). Coliphages were propagated in the appropriate host strain in TSB on a shaker platform (100 RPM) overnight at 36°C. Then, the broth cultures were vigorously mixed with fluorocarbon (Freon) for 2 min and then centrifuged at 2,600g for 15 min. Semi-purified virus supernatant was retained. Each strain was further purified by filtering using a 0.22 μm pore size syringe filter and the filtrate was retained for use as test coliphage. Host bacteria and coliphages were stored as stock at -80°C. Phage infectivity titers were determined by single agar layer plaque assay (SAL, EPA method 1602, 2001). For an inactivation experiment, each type strain of somatic coliphage was harvested in PBS as the top agar layer having confluent host cell lysis and the mixture was extracted with equal volume of Freon. After centrifugation at 2,600g for 15 min, the supernatant was archived and mono-dispersed by microporous polycarbonate filtration through 0.2 and 0.08 μm pore size filters that were pretreated with 0.1% Tween 80 solution and then rinsed with reagent water to remove excess Tween 80.

Survival of coliphage type strains of families in seeded water Survival tests were conducted using representative type strains of somatic coliphages representing each of the 4 major taxonomic groups. For each strain tested, virus stock was spiked into 40mL of test water to give an initial concentration of about 10^7-10^8 PFU/mL. A positive control sample for measuring the initial virus concentration in the spiked water at time 0 was taken and assayed for virus infectivity immediately after spiking. One aliquot of test water was held at room temperature (23-25°C), and one was held at refrigerator temperature (4°C). At each of a series of time intervals from 0 to 90 days, samples were taken and assayed for virus infectivity by SAL using the appropriate *E. coli* host. Duplicate samples were assayed at each time point.

Inactivation of somatic coliphages by the physical agents of UV radiation and heat A collimated beam UV apparatus (custom-made) emitting monochromatic UV radiation at 254 nm was used for UV inactivation experiments. A calibrated radiometer (International Light IL500) was used for measuring UV radiation in a 60×15-mm petri-dish. Purified and mono-dispersed somatic coliphage type strains in 5 ml volumes of PBS containing 10^5-10^6 PFU/ml in 60×15-mm petri-dishes were irradiated with the UV collimated beam system with slow stirring at room temperature. Average low-pressure UV lamp intensity (mW/cm^2) was measured and corrected to provide average incident irradiance. Target doses were calculated by the product of average irradiance and time. The average irradiance was calculated from the Lambert-Beer Law. Samples were taken from the UV irradiation system after calculated exposure times and their infectivity was determined. Also, purified and mono-dispersed

somatic coliphage type strains were used for a heat inactivation test in the following two conditions: Condition 1 = 55°C for 1 hour and Condition 2 = 63°C for 40 minutes.

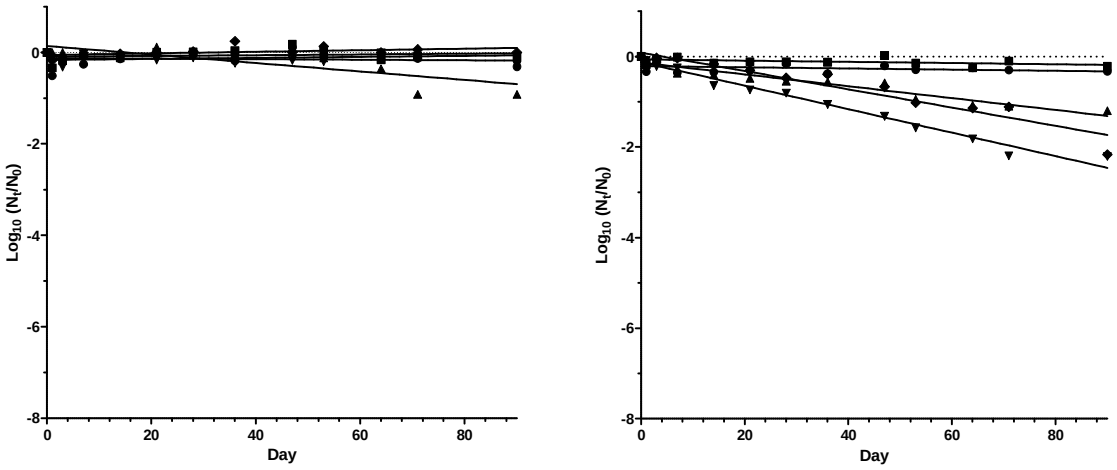
Statistical analysis Regression analysis was conducted for each type strain of somatic coliphage on their data for survival and inactivation using the GraphPad Prism 5 program (GraphPad, San Diego, CA) and SAS (8.2) program. Also, Excel 2003 (Microsoft Corp.) was used for survival and inactivation analysis by regression methods.

Results

Survival of coliphage type strains of families in seeded water Viruses were seeded at initial concentrations sufficient to observe 5-6 $\log_{10}$ reduction over time. Survival of somatic coliphage type strains in seeded water over 90 days at two temperatures is shown in Figure 2.1 and 2.2. In reagent water at 4°C (Fig. 2.1a), T1, T4, phiX174 and Lambda showed no infectivity titer reduction over 90 days. T7 showed approximately 1 $\log_{10}$ reduction over 90 days. At room temperature (Fig. 2.1b), the inactivation rates of each somatic coliphage strain differed. Somatic coliphage infectivity titer reductions over 90 days were T1 and T4 < 1 $\log_{10}$, Lambda and PhiX174 = 2 $\log_{10}$, and T7 = 1 $\log_{10}$. Regression analysis on reagent grade water at 4 and 25°C is shown in Figure 1 as a line and dots show the observed survival data of each somatic coliphage type strain over time. Inactivation of somatic coliphage type strains in reagent grade water was log-linear at both temperatures by regression anaysis. Regression analysis of reagent grade water at 4°C showed that infectivity of T7 declined by approximately 0.06 $\log_{10}$ per week and the slope was significantly non-zero (p=0.0016). However, the slopes of T1, T4, PhiX174, and Lambda were not significantly different from

zero on linear regression analysis. Regression analysis on reagent grade water at 25°C showed that T7 and Lambda declined by 0.1 $\log_{10}$ per week and PhiX174 declined by 0.2 $\log_{10}$ per week. The slopes of the regression lines for T7, Lambda and PhiX174 were significantly non-zero ($p<0.0001$). However, the slopes of T1 and T4 were not significantly different from zero.

a. b.

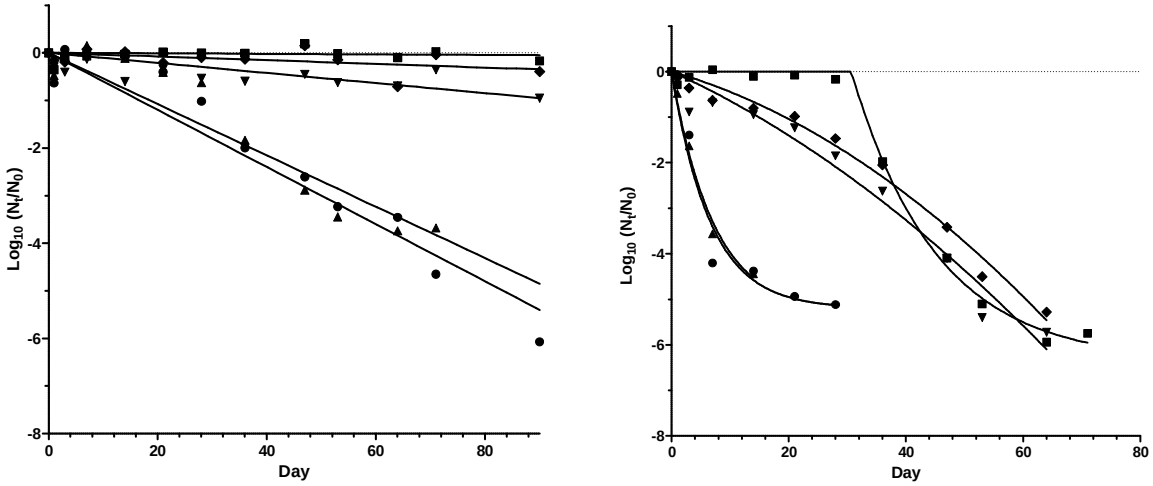


1. Figure 2.1 Log reductions (N_t/N_0) over 90 days in reagent water at temperatures of 4°C (a) and 25°C (b). Points =observed data; lines= predicted values from linear regression analysis (● : T1, ■ : T4, ▲ : T7, ▼: PhiX174,: ♦ Lamda).

Figure 2.2 shows the results for somatic coliphage type strain reductions in surface water. At 4°C (Fig. 2.2a), T4, PhiX174, and Lambda strains showed less than 1 $\log_{10}$ reductions over 90 days, while T7 and T1 showed approximately 4 $\log_{10}$ and 6 $\log_{10}$ reductions, respectively. For T1 and T7 at 4°C, there were progressive declines in infectivity that followed first order kinetics over 90 days. By linear regression analysis of infectivity data in surface water at 4°C, T1 declined by approximately 0.5 $\log_{10}$ per week and T7 declined by approximately 0.4 $\log_{10}$ per week. The slopes of the first-order regression lines

for T1 and T7 were significantly non-zero ($p<0.0001$). For PhiX174 and Lambda, regression analysis showed that inactivation rates of each strain were approximately 0.04 and 0.02 $\log_{10}$ per week, respectively. Also, the regression line slopes of PhiX174 ($p<0.0001$) and Lambda ($p=0.0092$) were significantly different from zero. However, the slope of the regression line for T4 was not significantly different from zero. As shown in figure 2.2b, there was a decline in the titer of all strains over 90 days at room temperature. After two months, all somatic coliphages tested showed 4-6 $\log_{10}$ reduction. However, compared to the other somatic coliphages tested, T1 and T7 were inactivated most rapidly in surface water at room temperature. T1 experienced 5 $\log_{10}$ reduction after 28 days, and T7 experienced 4 $\log_{10}$ reduction after 14 days. The inactivation kinetics in surface water at 25°C showed that rates of infectivity decline were not first order in all tested strains (Fig. 2.2b). For these conditions, kinetic analysis was done using non-linear regression models for all coliphage strains. For inactivation kinetics of T1 (R-square=0.9564) and T7 (R-square=0.9894), regression analysis was conducted using an exponential model with one phase of decay. Also, for T4, regression analysis was conducted using an exponential model with a plateau followed by one phase of exponential decay (R-square=0.9957). The decline of the infectivity of T4 started after one month and continued gradually during the survival test. There was no infectivity decline of T4 in the other conditions, except for surface water at 25°C. The microbial activity in surface water at 25°C may cause the decline of T4 infectivity and accelerate inactivation. For PhiX174 (R-square=0.9680) and Lambda (R-square=0.9845), regression analysis was done using a second order polynomial model. Compared to T1 and T7, T4, the infectivity of PhiX174 and Lambda declined more slowly over the time.

a. b.



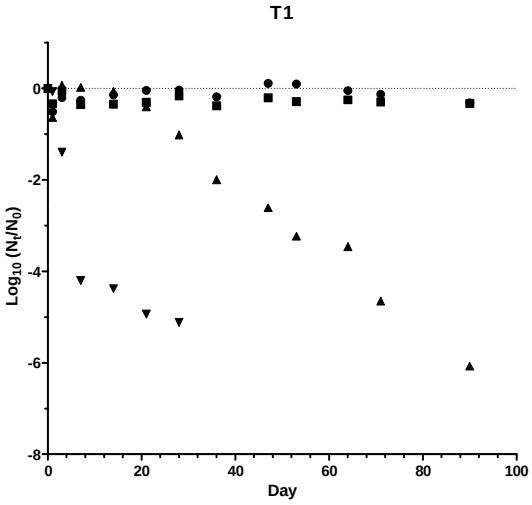
2. Figure 2.2 a. Log reductions (N_t/N_0) over 90 days in surface water at temperatures of 4°C (a) and 25°C (b). Points =observed data; lines= predicted values from linear regression analysis (● : T1, ■ : T4, ▲ : T7, ▼:PhiX174,: ♦ Lambda).

Figure 2.3 show a comparison of the reduction by strains for each water type (Reagent grade water and surface water) and temperatures (4 and 25°C) according to incubation times (days). Regression analysis was conducted using SAS (8.2) program to determine which variables were significant predictors of inactivation. Regression analysis was performed to determine if there were interaction effects between water type and temperature for each virus type. R-square values from regression analysis increased from 0.538 (model without interaction variable) to 0.854 (model with interaction variable) confirming that interaction between temperature and water type is a predictor of inactivation.

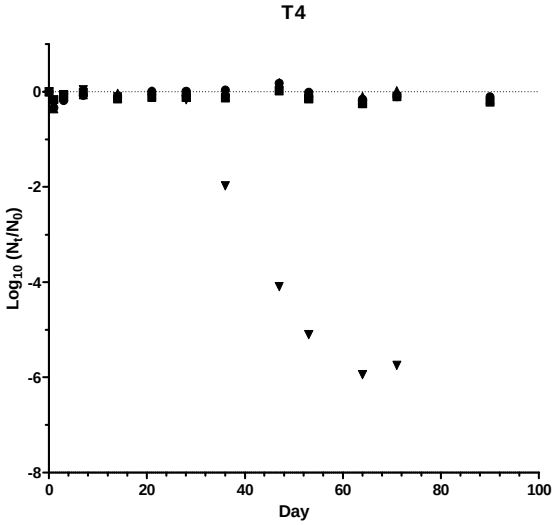
From this analysis, virus type, water type, temperature, and incubation time were significant predictors of inactivation ($p<0.0001$). Although virus type was a significant predictor overall, when each virus type is considered separately, PhiX174, Lambda, T1, and T7 were significant predicative variables ($p<0.0001$), whereas T1 was not significant

(p=0.3661). When interactions between individual virus types and water type were assessed, the interactions between water type and each individual virus were significant (For PhiX174, Lambda, T4, and T7 P<0.001, for T1 p=0.0286). When interactions between individual virus types and temperature were assessed, the interactions between temperature and each individual virus were not significant (p>0.05). Therefore, water type is a more important predictor than temperature for estimating virus reductions.

a

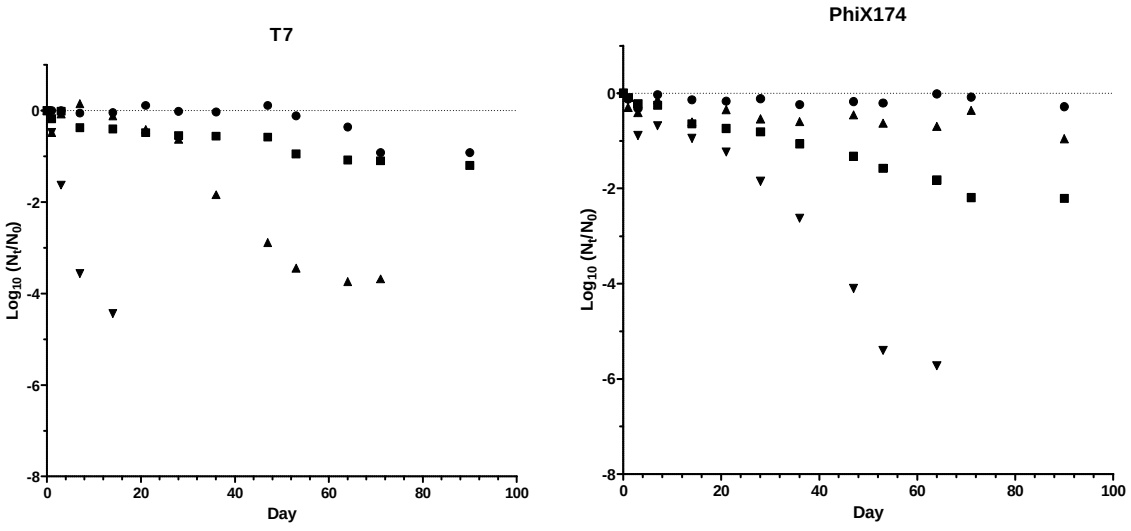


b

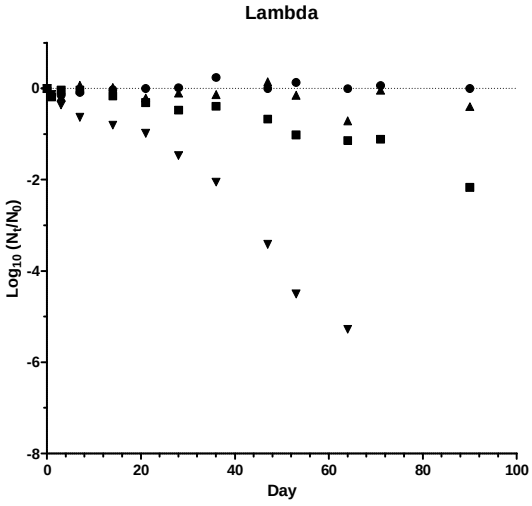


c

d



e



3. Figure 2.3 Log reductions (N_t/N_0) of T1 (a), T4 (b), T7 (c), PhiX174 (d) and Lambda (e) over 90 days (● : Reagent water at 4° C, ■ : Reagent water at 25° C ▲ : Surface water at 4° C ▼: Surface water at 25° C).

Table 2.1 provides the predicted times for 90%, 99%, 99.9%, and 99.99% (1, 2, 3 and 4 $\log_{10}$) reduction in days of all somatic coliphage type strains in both water types and at both temperatures by regression analysis. For all tested coliphage strains, the required time for 90% reduction in reagent grade water was longer than in surface water. Also, the time for

90% reduction was longer at 4°C than at 25°C for most coliphage strains in both water types. However for both T1 and T4 at both temperatures, the time required for 99.99% (4 $\log_{10}$) was over 1 year. At 4°C in surface water, strains T1 and T7 required 68 and 73 days for 99.99% (4 $\log_{10}$) reduction respectively, while the other three strains required over 1 year for 99.99% (4 $\log_{10}$) reduction. Also, there were differences among virus types in predicted times for infectivity reduction. At 25°C in surface water, the predicted times for infectivity reductions were more diverse among virus types. However, for 99.99% (4 $\log_{10}$) reductions at 25°C in surface water, T4, phiX174, and Lambda required longer times than T1 and T7. Also, at 4°C in surface water, these three strains required greater one year for 99.99% (4 $\log_{10}$) reduction, while T1 and T7 required only 68 and 73 days, respectively.

1. Table 2.1 Predicted times (days) for reduction of somatic coliphage type strain (T1, T4, T7, PhiX174 (φX174) and Lambda (λ)) infectivity at 4°C and 25°C in reagent grade water (a) in surface water (b).

(a)

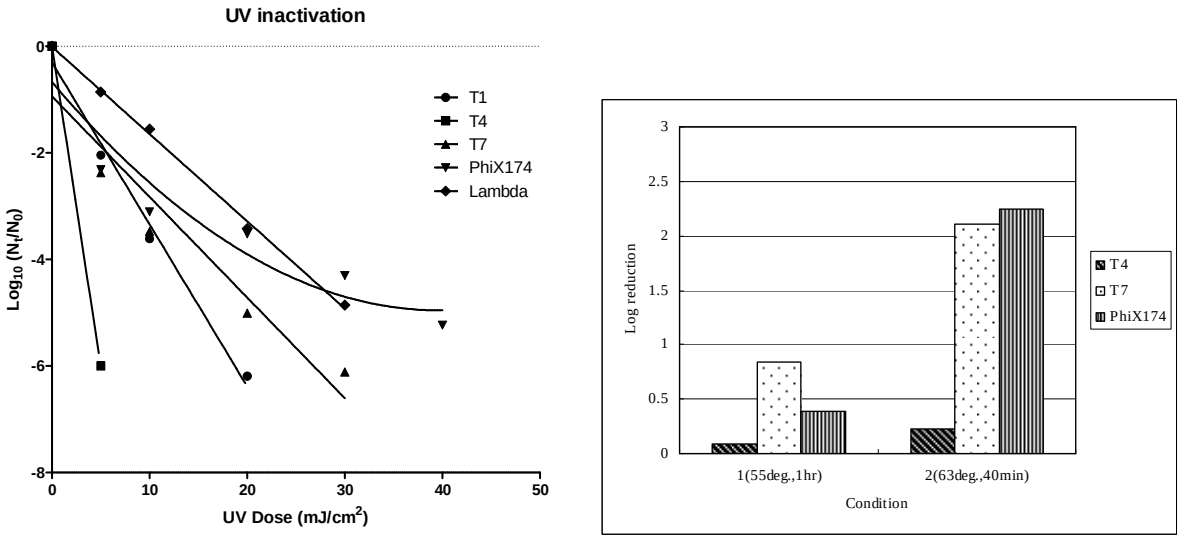
Reduction (Log10 (Nt/N0))	25°C					4°C				
	T1	T4	T7	φX174	λ	T1	T4	T7	φX174	λ
-1 (90%)	>365	>365	68	35	55	>365	>365	124	>365	>365
-2 (99%)	>365	>365	144	73	104	>365	>365	232	>365	>365
-3 (99.9%)	>365	>365	221	111	153	>365	>365	339	>365	>365
-4 (99.99%)	>365	>365	297	150	203	>365	>365	>365	>365	>365

(b)

Reduction (Log10 (Nt/N0))	25°C					4°C				
	T1	T4	T7	φX174	λ	T1	T4	T7	φX174	λ
-1 (90%)	2	34	2	15	19	22	>365	22	122	288
-2 (99%)	4	37	4	28	34	37	>365	38	285	>365
-3 (99.9%)	6	42	6	38	44	51	>365	57	>365	>365
-4 (99.99%)	9	47	10	48	53	68	>365	73	>365	>365

Inactivation of somatic coliphages by the physical agents of UV radiation and heat The coliphages with the slowest inactivation rates in water at 25°C were further tested to determine their inactivation kinetics by heat and UV light. Figure 2.4a shows the inactivation kinetics of five mono-dispersed coliphage type strains representing different taxonomic groups exposed to monochromatic UV radiation of 254 nm wavelength in a collimated beam apparatus. T4 (*Myoviridae*) showed high UV sensitivity, with greater than 6 $\log_{10}$ inactivation at a dose of less than 10 mJ/cm^2. Among tested strains, T4 strain has the largest genome size (~166kb) compared to the other strains (5~ 48kb) and is a double stranded DNA virus. These physical characteristics of T4 might influence the inactivation rate by UV radiation. T4 (*Myoviridae*), T1 (*Siphoviridae*), Lambda (*Siphoviridae*), and T7 (*Podoviridae*) showed log-linear regression kinetics, while phiX174 (*Microviridae*) showed non-log-linear regression kinetics (Second order polynomial model). Regression analysis showed that infectivity of T1, T4, T7, and Lambda declined by approximately 0.3 $\log_{10}$ per m J/cm^2, 1.2 $\log_{10}$ per m J/cm^2, 0.2 $\log_{10}$ per m J/cm^2, and 0.2 $\log_{10}$ per m J/cm^2, respectively. UV doses for 99% inactivation were 6 mJ/cm^2 for T1 and T7, 2 mJ/cm^2 for T4, 7 mJ/cm^2 for phiX174, and 12 mJ/cm^2 for Lambda (Table 2.2).

(a) (b)



4. Figure 2.4 Log_{10} inactivation (N_t/N_0) in buffered water by monochromatic UV radiation Points =Observed data; lines= predicted values from regression analysis (a) and inactivation of somatic coliphage by heat treatment with condition (b).

Inactivation of mono-dispersed preparations of some type strains of somatic coliphages by heat was tested at 55°C for 1 hour (condition 1) and 63°C for 40 minutes (condition 2). Condition 2, 63°C for 40 minutes, was more effective to inactivate somatic coliphages than was condition 1, 55 ^{o}C for 1 hour, with log_{10} reductions several times greater at the higher than at the lower temperature. T4, T7, and phiX174 experienced less than l log_{10} reduction at condition 1. At condition 2, T4 showed less than 1 log_{10} reduction while both T7 and PhiX174 showed >2 log_{10} reduction (Fig. 2.4b).

2. Table 2.2 Predicted UV dose (mJ/cm2) for decimal inactivation of T1, T4, T7, phiX174 and Lambda

Reduction Log_{10} (Nt/N0)	T1	T4	T7	phiX174[1]	Lambda
-1 (90%)	2.4	<1	<1	1.6	6.3
-2 (99%)	6	2	6	7	12
-3 (99.9%)	9	3	11	13	18

-4 (99.99%)	12	4	17	21	25

[1]: Non-linear regression analysis (second order polynomial model) was used to predict log inactivation. For the other strains, linear regression analysis was used to predict log inactivation

The results of studies comparing the different strains of somatic coliphages representing different families for their resistance against two physical agents, UV radiation and heat, showed trends in that T4 of the *Myoviridae* family was least resistant to UV radiation but most resistant to heat. Lambda of *Siphoviridae* family and PhiX174 of the *Microviridae* family showed higher resistance to UV radiation. PhiX174 has the smallest genome size (~5kb) compared to the other tested strains (42~166kb) and is a single stranded DNA virus, while the others are all double stranded DNA. Lambda has a relatively small genome size (~48kb) compared to the other double stranded DNA strains in this study. The genetic characteristics of PhiX174 and Lambda might contribute to their resistance to the physical stresses caused by UV radiation. The observation that PhiX174, Lambda, and T4 were relatively resistant to UV radiation or heat suggests that these viruses and their families may be among of the more persistent somatic coliphages in ambient waters subjected to physical environmental stressors.

Discussion

There are many aspects of somatic coliphage biology and ecology that need to be understood if they are to be used as viral indicators of fecal contamination in water. The results of this study show that for several factors, namely survival in water and when exposed to the physical inactivating agents of heat and UV radiation, different somatic coliphage strains representing different virus families can differ in response to exposure to these aquatic

conditions or inactivating agents. These results have important implications for the evaluation of these phages and their families as fecal indicator viruses. There are several criteria for the selection of an ideal indicator microorganism for detection of fecal contamination in water. One is that an ideal indicator should have survival characteristics similar to pathogens of interest.

The observations of comparative somatic coliphage survival in seeded waters suggest that *Myoviridae* as represented by T4, *Microviridae* as represented by phiX174, and *Siphoviridae* as represented by Lambda are potential candidates to serve as indicators of human viruses of fecal origin in water. Based on their slower inactivation rates in water over time, the representative strains of these three families were relatively persistent over time. At 4°C, their infectivity declined more slowly than did that of T1 (*Siphoviridae*) and T7 (*Podoviridae*). T4 (*Myoviridae*), PhiX174 (*Microviridae*) and Lambda (*Siphoviridae*), with less than l $\log_{10}$ inactivation over 90 days at 4°C in both reagent grade water and surface water. In surface water at room temperature, these three candidate strains representing their families also persisted longer than did T1 and T7. However, these three somatic coliphage strains started to decline in infectivity after one month in surface water, with a continuous infectivity decline over the remaining two months of the experimental period. For the first month, however, these three strains were much more persistent in water than were than T1 and T7. Also, when regression (SAS 8.2) analysis was performed to statistically determine if there were interaction effects between water type and temperature for each virus type, the interactions between water type and each individual virus were significant (For PhiX174, Lambda, T4, and T7 $P<0.001$, for T1 $p=0.0286$), while the interactions between temperature and each individual virus were not significant ($p>0.05$). Therefore, water type is a more

important predictor than temperature for estimating virus reductions. The water used for survival testing in this study was not sterilized, and contained viable microorganisms. The concentration of microorganisms in test water increased during the survival study, suggesting that the microbial activity in test water may affect the rates of viral inactivation. Although only one prototype strain in each family was investigated in this study, if these strains are representative of the survival of other members in their family, their families have potential as candidate somatic coliphage indicators of virological water quality.

It is recommended that somatic coliphages be directly compared to F+RNA and F+ DNA coliphages for their survival in water. The F+ coliphages are more commonly recommended candidate indicators for sewage contamination and enteric viruses in water, and previous studies have characterized their survival in different water matrices in comparison to the survival of human enteric viruses. Chung *et al*. (1993) showed that F+coliphages were inactivated faster than hepatitis A virus, poliovirus, and rotavirus in sea water in warm (summer time) conditions. Yahya *et al* (1993) compared the survival of bacteriophages MS-2 (F+ RNA; *Leviviridae* family) and PRD-1 (somatic coliphage; *Podoviridae* family) in groundwater, finding that PRD-1 was more persistent than MS-2 at higher water temperatures. Brion *et al* (2002) compared the inactivation kinetics of prototype strains of F+RNA coliphages in natural surface waters and showed that inactivation rates differed by strain. Of the F+ RNA phages MS2, GA, GB, F1, and SP, representing genogroups GI, GII, GIII and GIV, MS2 (GI) was inactivated most rapidly, declining by 7 $\log_{10}$ in two weeks (14 days), while the other F+RNA coliphages were inactivated completely after 36 days (about 5 weeks) in natural waters. Taken together, previous results suggest that F+ coliphages are inactivated more quickly than enteric viruses at higher water temperatures.

Based on the results of this present study as compared to the results of other candidate coliphages in previous studies, somatic coliphages are likely to be more persistent in water than F+ RNA coliphages. Therefore, based on their persistence, somatic coliphages may have advantages to F+ coliphages as more environmentally persistent indicators of enteric viruses in water.

In previous studies the *Myoviridae* family was abundant in human sewage and the *Siphoviridae* family predominanated in surface waters (Ackermann and Nguygen 1983; Pedroso and Martins 1995; Muniesa *et al.*, 1999). The results of this present somatic coliphage survival study are consistent with these findings if survival in water and wastewater is an important factor contributing to their presence. Previous studies have also explored the response of different F+ and somatic coliphages to disinfection processes, such as chlorination and UV irradiation. Duran *et al* (2003) found that isolates belonging to the *Siphoviridae* family were the most resistant to chlorination compared to enteroviruses and *E. coli*. However, in this present study, two *Siphoviridae* family type strains, T1 and Lambda, showed different inactivation kinetics in seeded water, suggesting that the *Siphoviridae* survival may vary among strains.

In this study, type strains of somatic coliphages showed relatively high UV sensitivity. Regression analysis on predicted UV dose for various $\log_{10}$ reductions (Table 2) showed that all type strains of somatic coliphages were inactivated by 4 $\log_{10}$ at doses lower than 25 mJ/cm^2. Based on comparisons to previous work with F+ RNA coliphages and adenoviruses, all somatic coliphage strains tested showed greater UV sensitivity than does MS2, a male-specific coliphage of the *Leviviridae* family (approximately 2 log reduction at a dose of 30

mJ/cm^2) (Shin *et al*., 2003), and Adenovirus 2, a member of the *Adenoviridae* family, (approximately 4 log reduction at a dose of 120 mJ/cm^2) (Shin *et al.*, 2005).

The taxonomic diversity of the somatic coliphage group makes it challenging to study their usefulness as viral indicator microorganisms. Even though all of the type strains representative of the different somatic coliphage families were not investigated in these studies on survival in water, the results of this study suggest that phages belonging to the *Microviridae, Myoviridae, and Siphoviridae* family are the most persistent in water. Also, compared to previous studies on the survival of F+ coliphages (Chung *et al*., 1993; Yahya *et al*., 1993; Brion *et al*., 2002), these somatic coliphage families also showed slower and less extensive inactivation at higher temperatures in environmental surface water. Further study of comparative survival and inactivation of individual families of somatic coliphages, including comparisons to human enteric virus survival, would provide better understanding of somatic coliphages as possible viral indicators of fecal contamination and human enteric viruses in environmental waters and such studies are recommended.

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CHAPTER 3 Molecular typing of somatic coliphages to determine their survival in environmental water

Abstract

A conventional, group-specific PCR method was developed to identify each of the 4 major taxonomic groups of somatic coliphages and used to classify individual isolates. Somatic coliphage survival in primary human sewage effluent was observed over time to further describe the behavior of somatic coliphages in environmental waters. Over time, the taxonomic makeup of the somatic coliphage population in sewage changes, with the *Microviridae* family becoming the most prevalent taxonomic group in the sewage population after several weeks. Based on their persistence and prevalence in environmental waters, phages belonging to the *Microviridae* family could serve as indicators for sewage contamination and possibly for human enteric viruses in sewage-contminated water.

Introduction

Due to the public health risks posed by enteric viruses in water, reliable indicators for the presence of these viruses in drinking and recreational waters have long been sought. Although there are culture and molecular methods available for the detection of enteric viruses in water, application of these methods is difficult, expensive and slow to yield results when attempting to simultaneous detect all types of enteric viruses.

Coliphages, viruses infecting *E. coli*, have been proposed as potential viral indicators because they are structurally similar to enteric viruses, abundant in sewage and readily detectable by relatively simple, rapid methods. Two major categories of coliphages, based on how they attach to infect host cells, are male-specific and somatic. Male-specific (F+) coliphages infect male or F+ strains of *E. coli* via attachment to F-pili, and somatic coliphages infect via direct attachment to receptors on the outer the outer surface of the cell. Male-specific coliphages have been primarily studied as potential viral indicators in water due to their structural similarity to many enteric viruses (Colford *et al*., 2007; Dore *et al*., 2000; Cole *et al*., 2003; Love and Sobsey, 2007). However, F+ coliphages have limitations due to their low occurrence and concentrations in feces, variable concentrations in sewage and rapid die-off in water (Love *et al*., 2007).

Somatic coliphages also have been proposed as possible alternative viral indicators in sewage and water (Kott, 1966, Dhillon *et al*., 1970; Dhillon and Dhillon, 1972; Moce-Llivina *et al*., 2005; Muniesa *et al*., 2007; Jofre, 2008). Somatic coliphages are a heterogeneous group encompassing four virus families: *Myoviridae, Siphoviridae, Podoviridae, Microviridae,* containing several genera (The Bacteriophage, 2nd edition, chapter 2, 2006). Somatic coliphages are an extremely diverse viral group, with variations in DNA content, size, structure, and life cycle and include tailed, double-stranded DNA phages of the order *Caudoviriales* and those of small size and cubic symmetry, containing single-stranded DNA (*Microviridae* family).

Previous studies that have assessed the use of somatic coliphages as indicators of enteric viruses have not systematically taken into account the taxonomic diversity within the group itself. The somatic coliphages found in fecal sources have not been genetically characterized

despite their abundance, and it is not clear if the relationships between the presence of somatic coliphages and enteric viruses in water may differ by genetic group or strain of somatic coliphage. Many studies of somatic coliphages as viral indicators treat somatic coliphages as homogeneous, even though they are potentially genetically diverse. Because of their diversity, the presence and behavior of somatic coliphage in environmental water systems is probably also diverse based on strain and family. Therefore, it is understandable why somatic coliphages as a broad and diverse group may have little significant association with enteric virus presence in water (Hot *et al*., 2003).

In order to assess the value of somatic coliphages as potential indicators of enteric viruses of human health risk, their persistence in the environment needs to be evaluated by accounting for the taxonomic groups in order to identify those that are the best indicators. Even though somatic coliphages are routinely detected in human sewage (Moce-Llivina *et al*., 2005), the extent to which individual families and strains of somatic coliphages are potential indicators of viral contamination sources in environmental waters is unknown in part because the possible presence and survival of different taxonomic groups has not been determined.

To determine whether somatic coliphages are useful indicators for human enteric viruses in water, the prevalence and comparative environmental persistence of members of the somatic taxonomic group needs to be evaluated. Characterization of the relative abundance in human waste of specific virus families within the broad somatic coliphage group may identify individual families or strains that are more feces-specific than the somatic coliphage group as a whole, much as *E. coli* is a feces-specific organism within the larger coliform bacteria group (Dockins *et al*., 1978). This approach is supported by previous studies that show the *Myoviridae* family to be the predominant somatic coliphages in human

sewage, and the *Siphoviridae* family to be the most common somatic coliphage group in surface waters (Ackermann *et al*., 1983; Pedroso *et al*., 1995; Muniesa *et al*., 1999). Comparative survival studies using different coliphage strains representative of each virus family can also help determine which coliphages are the most environmentally persistent, a key criterion for an ideal indicator.

In order to examine the importance of taxonomic identity in studies of somatic coliphages as indicators, methods are needed to genetically characterize these phages. Rapid nucleic acid-based molecular methods are promising for not only genetically characterizing somatic phages, but for routine environmental monitoring for the presence of these phages in water and wastewater.

In order to identify a candidate somatic coliphage family for use as an indicator for the presence of human enteric viruses in surface water, we evaluated the comparative persistence and prevalence of the taxonomic group(s) among the somatic coliphages using newly developed nucleic acid-based molecular methods for the detection of specific somatic coliphage groups.

Materials and Methods

Coliphage propagation Type strains of somatic coliphage used in this study were PhiX174 (*Microviridae*), T4 and Mu (*Myoviridae*), T7 and N4 (*Podoviridae*), T1, Lambda and HK97 (*Siphoviridae*). Phages and their bacterial hosts were obtained from the Felix d'Hérelle Reference Center for Bacterial Viruses, University Laval in Canada. Bacteriophages were propagated in the following *E. coli* hosts: T1, T4, and T7 in *E. coli* B, and PhiX174, Lambda, HK97, and N4 in *E. coli* C, *E. coli* K12S Lederberg, *E. coli* Y mel mel-1 supF58, and *E. coli*

W3350, respectively. Each host strain was grown in tryptic soy broth (TSB; Difco). Coliphages were propagated in the appropriate host strain in TSB on a shaker platform (100 RPM) overnight at 36°C. Overnight broth cultures were vigorously mixed with fluorocarbon (Freon) for 2 min and then centrifuged at 2,600g for 15 min. Semi-purified virus supernatant was retained as virus stock. Each strain was further purified by filtering using a 0.22 μm pore size syringe filter. Host bacteria and prepared coliphage stocks were stored at -80°C. Phage titers were determined by single agar layer plaque assay (SAL, EPA method 1602, 2001).

Survival of wild-type somatic coliphage strains in water Wild-type somatic coliphage survival in primary sewage effluent was determined for primary effluent from the Orange County Water and Sewer Authority (OWASA), the wastewater treatment plant serving Chapel Hill, NC. Primary effluent was collected and transported to the laboratory. A control sample to measure the initial virus concentration at time 0 was taken and assayed immediately. One aliquot of effluent was held at room temperature (23-25°C), and one was held at refrigerator temperature (4°C). Samples were taken and assayed for virus infectivity by SAL using *E. coli* CN-13 as a host at the time intervals of 10, 15, 29, 47, and 65 days. Duplicate samples were assayed at each time point.

At each time point, a representative number of somatic coliphages plaques (20-50) were chosen randomly (considering different plaque sizes, morphologies, and not spatially biased in one location or plate) from SAL plates for characterization. Plaques were picked by using the tip of a micropipettor (20-200ul capacity) set at 100ul volume, suspended in 100ul of TSB and assayed using a spot plate technique with individual plaques in each spot counted to determine sample titer. Individual plaques from each spot were picked, re-suspended, and re-

enriched for 24 hours in TSB using host bacterium *E. coli* CN-13. After incubation, the enriched phage isolates were filtered to remove cell debris using 0.45um pore size filters, and frozen at -80°C for further characterization.

Conventional PCR for family-level identification To determine which families of somatic coliphages were isolated from primary sewage effluent, oligo-nucleotide primers targeting the members of each somatic coliphage family were developed using bioinformatics tools. Sequence analysis of all four families was conducted by applying bioinformatics tools to the full genome sequences of strains from the *Myoviridae* (9 strains), *Microviridae* (43 strains), *Podoviridae* (8 strains), and *Siphoviridae* (9 strains) archived in the National Center for Biotechnology Information (NCBI) website. Sequence analysis was done using Vector NTI (ver.10, Invitrogen), MEGA (version 4, Tamura *et al*., 2007), and Jalview (version 2, University of Dundee). Multiple alignments were carried out with ClustalW2, and blast searches of protein-protein in the *Myoviridae* and *Siphoviridae* families were conducted to find conserved protein regions among strains in each family to target with family specific primers.

Optimizing family-specific conventional PCR conditions Positive control coliphages from each family were used to optimize PCR conditions. PhiX174 (*Microviridae*), T4 and Mu (*Myoviridae*), T7 and N4 (*Podoviridae*), T1, Lambda and HK97 (*Siphoviridae*) were used as positive controls for each family. Viral DNA was extracted from stocks of these positive control coliphages using the QIAamp viral mini kit (QIAQEN Inc.). After serial

dilution of positive control DNA, PCR was carried out using different sets of amplification conditions, including different annealing temperatures and cycle numbers.

Detection limit of group-specific conventional PCR To determine the detection limit of the group-specific conventional PCR method, conventional PCR and plaque assay (SAL) was applied to serial dilutions of all positive somatic coliphage strains. Somatic coliphage stocks from each family were serially diluted in ten-fold, coliphage DNA was extracted from each dilution, and the optimized conventional PCR procedure was applied to the extracted DNA. The viral DNA was not detected in the PCR reactions past a certain dilution point, which taken as the detection limit endpoint of plaque assays (SAL) of somatic coliphage strains.

Sequencing of positive samples and phylogenetic analysis For further characterization of somatic coliphage environmental isolates from primary effluent, PCR products were purified (QIAquick PCR Purification kit; QIAGEN Inc.) and sequenced at the Genome Analysis Facility (University of North Carolina, Chapel Hill). Multiple sequence alignments and clustering of the sequenced isolates along with *Microviridae* full genome sequences from NCBI website (http://www.ncbi.nlm.nih.gov/) were conducted with the ClustalW2 program at the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/). Phylogenic analysis was conducted using the Neighbor-Joining method (Saitou *et al.*, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was displayed next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method as the substitution model in

nucleotide category in the MEGA4 program (Kimura, 1980). Phylogenetic analyses were conducted in MEGA4 (version 4, Tamura *et al*., 2007).

Results

Survival of somatic coliphages from sewage effluent Table 3.1 shows the concentrations of total somatic coliphages (all genogroups) in sewage samples over time at 4°C and 25 °C. The concentrations of total somatic coliphages decreased over time at both temperatures. However, the decline over time at 4°C was less than at 25°C. At both temperatures, some phages underwent a decline after day 28. Also, Table 3.1 shows the number of somatic coliphage isolates collected at each time point. After day 65, the concentration total of somatic coliphages at 25°C had declined to 0.1 PFU/mL, so only three somatic coliphage isolates were collected at this time period. From this time-course study of somatic coliphage survival in primary sewage effluent incubated at 25°C and 4°C, a total of 275 presumptive somatic coliphage isolates were collected and archived for subsequent taxonomic characterization.

3. Table 3.1 Survival of somatic coliphages in primary effluent sewage over time, and number of somatic coliphage isolates as picked plaques enriched at each sample time point at temperatures of 4 °C and 25 °C

	Temperature (°C)	day0	day10	day15	day29	day47	day65
Change of titer (Unit: PFU[2]/ mL)	4	400	170	141	23	N/A	32
	25	400	N/A	67	6	10	0.1
Number of Isolates	4	48	30	29	26	N/A	47
	25		N/A	30	26	36	3

[1]: N/A: data not available
[2]: PFU: Plaque Forming Units

Primer design and optimization for group-specific PCR of coliphage families Table 3.2 shows the virus families divided into subgroups, the primer target region, and the approach used to find the conserved regions by nucleotide or protein level analysis. After multiple alignment analysis by ClastalW2 tool, strains were grouped by conserved regions of nucleotides or proteins in each family to find candidate target regions for primers.

4. Table 3.2 Strains and target regions for somatic coliphage primer design

Family	Subgroup	Strains	Target	Approach
Myoviridae	T4 set	JS98, RB69, Phi1, RB49, **T4**[1]	major head protein (gene 23)	protein
	Mu set	**Mu**[1], P1, P2, Wphi	tail fiber gene (MUP49)	
Microviridae	-	**PhiX174**[1] and 43 strains	-	nucleotide
Siphoviridae	HK set	HK022, **HK97**[1], BP-4795	cII protein	protein
	JK set	JK06, RTP, TLS, **T1**[1]	tail fiber protein	
	Lambda set	**Lambda**[1], N15	B gene	
Podoviridae	933 set	933W, VT2, HK620, phiV10	933Wp09, hkaG gene	nucleotide
	K1F set	K1F, T3, **T7**[1]	CKV1F_gp34	
	N4	**N4**[1]	-	

[1]: Available positive control for subgroups

The candidate taxonomic group-specific primers designed by this process are shown in Table 3. The *Microviridae* and the *Podoviridae* families show conserved regions for primer design in multiple alignment analysis at the nucleotide sequence level. The *Myoviridae* and the *Siphoviridae* families showed greater diversity at the gene level, so a protein level approach was needed to design subgroup-specific primers. The subgroup was chosen based on the conserved protein region identified in each family. The primer location of the family was selected within subgroup multiple alignment of the target group (Table 3.3).

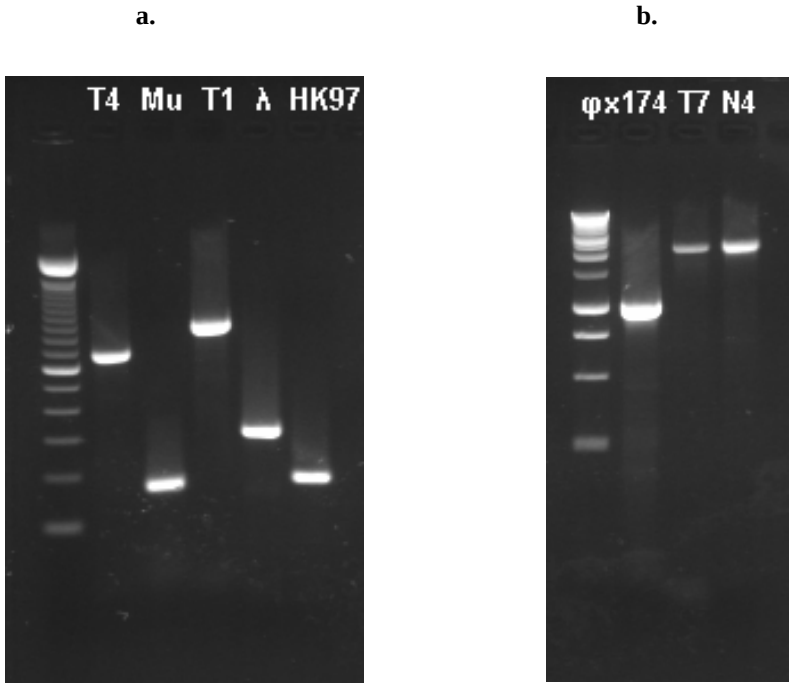
5. Table 3.3 Group specific primers for identification of environmental somatic coliphages

Family	Group	Candidates Primer sequences (5' - 3')	Size(bp)	Location[1]
Myoviridae	T4 set	GATATTTGTGGYGTTCAGCC (FW)	704	334-354
		GTCAAATACACCAGCTTTAGAACC (RV)		1014-1038
	Mu set	GAAAACGACTCAATCCTTGCC (FW)	171	2078-2099
		TCATCAGGTCTTTTGTTGTGG (RV)		2228-2249
Microviridae	-	GCTGCCGTCATTGCTTATTATGTTC (FW)	1039	2965-2990
		GYTAYCGBMMCATYAAYTAHTCACG(RV)		3979-4004
Siphoviridae	HK set	CACAGCGAGAAATTGATCGC (FW)	177	29-48
		CTAATCGGACTGATGTCTG (RV)		188-206
	JK set	GYGAYCAGATGGTTCC (FW)	878	993-1008
		CAATRTCYTCYTARTTG (RV)		1866-1871
	Lambda set	TGGGCGTACTTTATGGGGCG (FW)	307	1177-1196
		CGGACCTGCTGGGCAAAAAT (RV)		1465-1484
Podoviridae	933 set	GCAATACATCAAACGCCG (FW)	488	287-304
		GCGAATGCCAGCGGCG (RV)		760-775
	K1F set	TGGAAGCCCGTGAGAC (FW)	2110	33350-33366
		GCAGCGTCAATCGCTCGG (RV)		35442-35460
	N4	GCACATGCAGAATAAGGTTG (FW)	2285	2397-2417
		CCATTAGTAACACCATCTGC 20 (RV)		4662-4682

[1]: Location refers to the bp coordinates or amino acid coordinates within the conserved gene in all Families except *Microviridae* family and K1F set in *Podoviridae* family, where the coordinates refer to the genomic position

Once primers were selected, PCR conditions were optimized by comparing the results of varying annealing temperatures and amplification cycles. The PCR conditions that yielded a gradual decrease in the band thickness with serial dilution were selected as optimal and used for further characterization of isolates. For the *Myoviridae* family, optimized PCR conditions were: 95°C - 5min, [95°C - 30sec, 58°C - 30sec, 72°C - 30sec] x 40 cycles, 72°C - 10min, 4°C - 10min. For the *Microviridae* group, optimized PCR conditions were: 95°C - 5min, [95°C - 30sec, 61°C - 30sec, 72°C - 30sec] x 40 cycles, 72°C - 10 min, 4°C - 10min. For the HK97 and Lambda subgroup in *Siphoviridae* family and N4 in *Podoviridae* family, optimized PCR conditions were: 95°C - 5min, [95°C - 30sec, 55°C - 30sec, 72°C - 30sec] x

40 cycles, 72°C - 10min, 4°C - 10min. For the JK subgroup of *Siphoviridae* and K1F subgroup of *Podoviridae*, the optimized annealing temperatures were 51°C and 63°C, respectively. The other conditions were identical across subgroups. Figure 3.1 is an electrophoresis agarose gel stained with ethidium bromide showing all positive controls amplified at their optimized conditions. Cross-reaction or non-specific amplification within the primers of each subgroup and family was not found for any of the virus taxonomic groups studied.



5. Figure 3.1 Optimized PCR results with positive control somatic coliphage group specific primers in each family: For T4 (704bp), Mu (171bp), T1 (878bp), λ (Lambda, 307bp), and HK97 (177bp), 100bp was used as molecular weight marker (a). For ΦX (PhiX174, 1039bp), T7 (2110bp), N4 (2286bp), 1000bp laddar was used as molecular weight marker (b).

Somatic coliphage family identification using family-specific conventional PCR All environmental isolates collected from sewage survival experiments were tested using family specific primers and the optimized PCR conditions described above. As shown in Table 3.4,

the taxonomic diversity of the somatic coliphage population in environmental samples changes over time. At day 0 and day 1, 2 of the 4 somatic coliphage families were detected among isolates. In samples from days 47 and 65, only 1 of the 4 families was detected, although not all strains within the family were identified. At later time points, the *Microviridae* family became most prevalent in the somatic coliphage population. At day 47 (31 positive/36 isolates) and day 65 (3 postive/3 isolates) at 25°C more isolates were from the *Microviridae* family than from other families (Table 3.4). These results suggest that the *Microviridae* family is the most prevalent and persistent somatic coliphage group in sewage-contaminated environmental waters under the tested conditions. There are relatively few *Microviridae* among the Day 1 samples but many more *Microviridae*-positive samples at the Day 65. In addition, there were three samples positive for *Myoviridae* at Day 0-1 and at day 29, with but no *Siphoviridae*- or *Podoviridae*-positive environmental isolates (Table 3.4). On day 0, the families of the majority of somatic coliphage isolates (approximately 70%) could not be determined by the group-specific conventional PCR method developed. The archived strains of each family from the NCBI website were enterobacteriophage with full genome sequences not with partial genome sequences. The developed group-specific PCR methods could detect the strains of each family described in table 3.2. However, many strains in each family with partial genome sequences could not be detected by this method. It is possible that many of unidentified isolates could be from one of the strains with only partial genomes available in the database. Alternatively, this lack of detection could be caused by the detection limit of conventional PCR methods. It is possible that without further propagation to enrich them, the concentration of some isolates is not sufficient to exceed the lower

detection limit of conventional PCR assay (Table 3.5). Nevertheless, the somatic coliphage population has greater diversity in samples from day 0 than the samples of later time points.

6. Table 3.4 Family presence among somatic coliphage isolates as a function of sample incubation time based on application of candidate family specific primers for PCR analysis

Days	0-1	29(25°C)	47(25°C)	65(4°C)	65(25°C)
No. *Microviridae* Positives/Total No. Isolates	10/48	22/26	31/36	31/47	3/3
No. *Myoviridae* Positives/Total No. Isolates	2/48	1/26	0/36	0/47	0/3
No. *Siphoviridae* Positives/Total No. Isolates	0/48	0/26	0/36	0/47	0/3
No. *Podoviridae* Positives/Total No. Isolates	0/48	0/26	0/36	0/47	0/3

Detection limits of group-specific conventional PCR The lower detection limits of group-specific conventional PCR of positive strains of each somatic coliphage family was determined, and detection limits ranged from 0.4 PFU to 2×10^5 (Table 3.5).

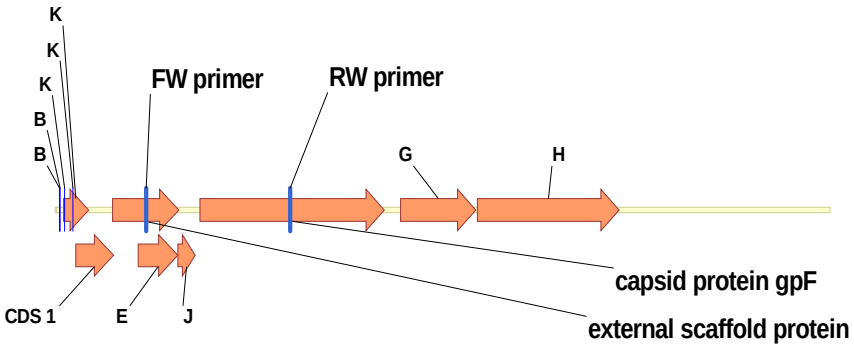
7. Table 3.5 Detection limit of each positive strain in four somatic coliphages families

Family	Primer set	Phage strain	Limits of detection (plaque forming unit per 10ul DNA template)
Myoviridae	T4	T4	2.8E+03
Microviridae	PhiX174	PhiX174	3.0E+02
Siphoviridae	HK	HK97	8.0E-01
	JK	T1	2.4E+03
	Lambda	Lambda	4.0E-01
Podoviridae	K1F	T7	8.0E+03
	N4	N4	2.0E+05

* Group-specific conventional PCR was applied to tenfold serial dilution of each strain representing each subgroup of a somatic coliphage family

Sequencing All PCR-positive environmental isolates from primary sewage effluent were sequenced to further characterize the genetic properties of these *Microviridae* and *Myoviridae* family members. All *Myoviridae* positive isolates mapped to T4 in the NCBI

blast database. The best blast match for the positive isolates in the *Microviridae* family mapped to ID1, Enterobacteria phage PhiX174 *sensu lato*. Figure 3.2 shows the sequenced region for the *Microviridae* family. The sequenced *Microviridae* positive isolates were clustered to better describe the phylogenetic relationships among them. Figure 3.3 shows the clustering of the *Microviridae* positive isolates from primary sewage effluent. As described in Rokyta *et al*. (2006), the *Microviridae* were grouped based on genome analysis into three subgroups: PhiX174-like, G4-like, and Alpha-3 like. Those three representative strains of this family and ID1 were included in clustering. Most of isolates were clustered with the PhiX174-like group.



6. Figure 3.2 Genome organization diagram representing the location of primers and the coding sequences of PhiX174 FW (forward) primer and RW (reverse) primer

7. Figure 3.3 Clustering of sequencing results of *Microviridae* family positive isolates from the survival test using ClustalW2 and MEGA (Version4) program. Dots show the positive controls which were included in the alignment and clustering. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches

Discussion

This study demonstrated that one particular somatic coliphage family, the *Microviridae*, is a potential viral indicator based on its presence, persistence and increased abundance over time in stored sewage effluent. Most studies to investigate somatic coliphage as viral indicators treated them as a single microbial group, even though they are taxonomically heterogeneous and highly diverse (Kott, 1966, Moce-Llivina *et al*., 2005; Muniesa *et al*., 2007; Jofre, 2008). Although previous evidence suggests that there is a lack of correlation of enteric viruses with somatic coliphages taken as a whole group (Hot *et al*., 2003), an individual family of somatic coliphage could potentially have a significant association with the occurrence of enteric viruses in water. Also, the developed conventional PCR method for specifically identifying members each somatic coliphage family could be used as a tool for characterizing coliphages of environmental waters by detecting the individual members of each somatic coliphage family in a total collection of isolates.

The isolates from the survival study of somatic coliphages in wastewater treatment plant primary sewage effluent were classified using optimized conventional group-specific PCR amplification methods for each individual family. A total of 160 somatic coliphage isolates were used to investigate the genetic diversity of somatic coliphage environmental isolates from primary sewage effluent. The *Microviridae* family was the most prevalent among the isolates from sewage effluent. In addition, the proportion of isolates belonging to the *Microviridae* family increased over time at 25°C. This result suggests that somatic coliphages of the *Microviridae* family present in sewage are the most persistent member of the somatic coliphage population. However, further studies are needed to determine if this family might consistently be the more abundant under different conditions in environmental waters.

In this study, the majority of identifiable somatic coliphages isolates from primary sewage effluent belonged to the *Microviridae* and *Myoviridae* families; no isolates were identified from the *Siphoviridae* or the *Podoviridae* families. Further studies are needed to determine if these results are characteristic of the somatic coliphage make-up of primary sewage effluent or if there could be differences in those families present based on regional and seasonal effects.

It is also possible that the observed results are not truly representative of the somatic coliphage population because the conventional group-specific PCR method is not able to detect all members of the *Siphoviridae* and the *Podoviridae* families. There may have been members of these families present in the primary effluent that were not detected by the primers used. The representative somatic coliphage family type strains used in this study for finding conserved regions in each family were complete genomes which were archived from NCBI website. Partial genomes present in the database were not used for the development of the family group-specific PCR. Therefore, it is possible that all strains in a family were not detected in this method because the strains were not sufficiently representative of the genetic diversity of the family. It is also possible that the lower detection limit of the PCR was not low enough to detect the low numbers of somatic coliphages in the samples subjected to PCR, despite enrichment. The lower detection limits of PCR ranged from as low of 0.4 PFU per reaction for the Lambda subgroup of the *Siphoviridae* family to as high as 2×10^5 PFU per reaction for the N4 subgroup of the *Podoviridae* family. The possiblity that the lower detection limit of PCR amplification is related to the size (bp) of the target amplicon was tested using Spearman rank correlation analysis and found to be positive ($r = 0.82$) significant ($p = 0.0341$). Lack of PCR amplification and identification of enriched coliphage

isolates has been reported by others in our laboratory, and the reasons for such findings remain uncertain. In addition, positive control coliphages were not available to optimize the PCR conditions for the subgroup of the 933 set in the *Podoviridae* family. Therefore, the selected candidate primers of the 933 set might not detect all possible members of the *Podoviridae* family.

Not all somatic coliphage isolates were identified to the family level from the sewage effluent survival test, as some isolates could not be genetically classified. However, the *Microviridae* family appears to be both most persistent and most abundant of the identifiable coliphage isolates of the 4 families detectable in the somatic coliphage population of primary sewage effluent. These results suggest that the *Microviridae* family could serve as a candidate somatic coliphage viral indicator based on their observed abundance and persistence as observed in the somatic coliphage time course study of primary sewage effluent.

Previous studies suggest that somatic coliphages as a broad group might not correlate with the occurrence of human enteric viruses in water. However, the results of present study provide the opportunity to determine if an individual somatic coliphage family such as the *Microviridae* might exhibit an association with human enteric virus contamination in environmental waters and hence could serve as a human enteric virus indicator system. However, further studies are needed to determine if there is such an association between the *Microviridae* and the human enteric viruses in sewage and sewage-contaminated waters.

Previous studies of somatic coliphage typing were based on electron microscopic examination of viral particle morphology (Muniesa *et al.*, 1999; Duran *et al*, 2002). Such electron microscopic analysis is usually tedious, slow and sometimes unreliable. Therefore,

the new nucleic acid molecular-based typing methods for somatic coliphage family detection developed in this study have the potential to provide a more convenient and more reliable analytical approach to understanding the genetic composition of environmental somatic coliphages. Further developing and applying nucleic acid-based detection and characterization methods will assist future studies to evaluate somatic coliphages as enteric viral indicators for water quality assessments. These newly developed methods can be used to expand the body of evidence on the utility of somatic coliphages as viral indicators by applying them in future studies of virological water quality and health. The optimized conventional group specific PCR methods developed in this study make possible further investigation of the occurrence of the somatic coliphage *Microviridae* family and human enteric viruses to gain a better understanding of their relationships and human health risk predictability in environmental waters.

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CHAPTER 4 Development and evaluation of real-time PCR and antibody-based immune-screening methods for rapid detection of somatic coliphage groups as viral indicators

Abstract

The need for a reliable viral indicator for estimating fecal contamination in water is increased by industrial development and population increases that lead to water quality degradation from growing and poorly managed sources fecal contamination. In addition to useful and reliable viral indicators, the need for a rapid detection method for estimating them in environmental waters has also been recognized and pursued in previous studies investigating virus detection methods. A possible viral indicator for environmental water is the *Microviridae* family of somatic coliphage, which was identified as possible candidate viral indicator group in a previous chapter.

In this study, two approaches are considered for rapid methods to detect somatic coliphages at the family level: real-time PCR as a molecular genetic based method and Culture, Latex Agglutination, and Typing (CLAT) assay as an antibody based method. The two methods were applied to environmental isolates from different water matrices: sewage effluent and seawater, for detecting and characterizing the families of somatic coliphages in environmental waters.

Developing new and rapid nucleic-based detection and antibody-based characterization methods will assist in future studies to evaluate somatic coliphages as viral indicators for

water quality assessment. These rapid detection methods will also make rapid water quality assessment possible if somatic coliphages are verified as reliable indicators of viral contamination in environmental waters. The results of this study show that PCR-based and antibody based methods can be used successfully to identify and classify somatic coliphages in water and human sewage, and can be used to expand the body of evidence on the utility of somatic coliphages as viral indicators in studies of water quality and health.

Introduction

The importance of reliable microbial indicators for estimating fecal contamination in water is increasing as water pollution resulting from the growth of both industries and human populations increase. In addition to reliable microbial indicators for assessing water quality, there is also great interest in rapid detection methods for indicators such as *E. coli* or other fecal bacteria. There has been a large amount of research into rapid and simple detection kit development for bacterial indicators, and several kits are now available for detecting those indicators in formats that are easy to use. However, such rapid methods for viral indictors lag behind those for bacteria.

Bacteriophages have been proposed as useful viral indicators in different environmental waters and for water treatment processes (Armon, 1993; Colford *et al*, 2007, Cole *et al*, 2003, Dore *et al*, 2000, Havelaar *et al*., 1993, Jofre *et al* 1995, and Lovelace *et al*., 2005). Male-specific coliphages and *Bacteroides fragilis* have been studied as possible indicators for estimating human enteric virus contamination. Rapid detection methods for candidate viral indicators have previously been investigated using several approaches (Kir *et al*., 2007, Love *et al*, 2007, Sim *et al*., 1995, Stanek *et al*., 2000, and Wentsel *et al*., 1981) including a

molecular approach using real-time PCR and an antibody based approach using Culture, Latex Agglutination, and Typing (CLAT).

In previous research individual somatic coliphage families were investigated as a candidate human enteric virus indicator in terms of their persistence and prevalence in environmental waters (Lee, unpublished data). If somatic coliphages are found to be useful viral indicators, culture methods for their detection still typically take at least one day to yield results. There are many situations where rapid detection and quantification of indicators such as a somatc coliphage are desirable if not essential. For example, because drinking water moves through the distribution systems to consumers within minutes to hours of leaving treatment facilities, results from current assays will lag behind actual drinking water contamination events before the water reaches consumers. Thus, there is a need for rapid detection methods for these viruses for timely management decisions about the microbial quality of water.

To detect somatic coliphages in water, culture based methods such as single agar layer, double agar layer, and enrichment methods (EPA 1601 and 1602, 2001) are used with an *E. coli* host, requiring overnight incubation to produce results such as plaques or lysis zones. However, to make timely decisions about water quality, same day detection of fecal virus contamination is highly desirable from a public health and drinking water treatment management viewpoint. Rapid detection methods for male-specific coliphages have previously been demonstrated and applied (Kirs *et al*., 2007; Love *et al*., 2007). It is hypothesized that the same rapid methods used for male-specific coliphages could be applied to rapid detection of somatic coliphages.

Real-time PCR is used to detect a wide variety of microorganisms in environmental waters in a relatively short time compared to culture based methods. Its rapidity, simplicity, and sensitivity make it an advantageous method for application to water quality monitoring. The specific probes and primers for amplification can increase the sensitivity and specificity of detection of target coliphages. In this study, the highly conserved regions in the *Microviridae* family of somatic coliphages were targeted for real time PCR amplification. The development of a real-time PCR method based on the conserved region of the candidate viral indicator family would make it possible to assess the water quality in a comparatively short time compared to typical culture based methods. In addition, the quantification of the *Microviridae* family is possible using qPCR after producing standard relationships between coliphage number and Ct value developed for positive control type strains from this family.

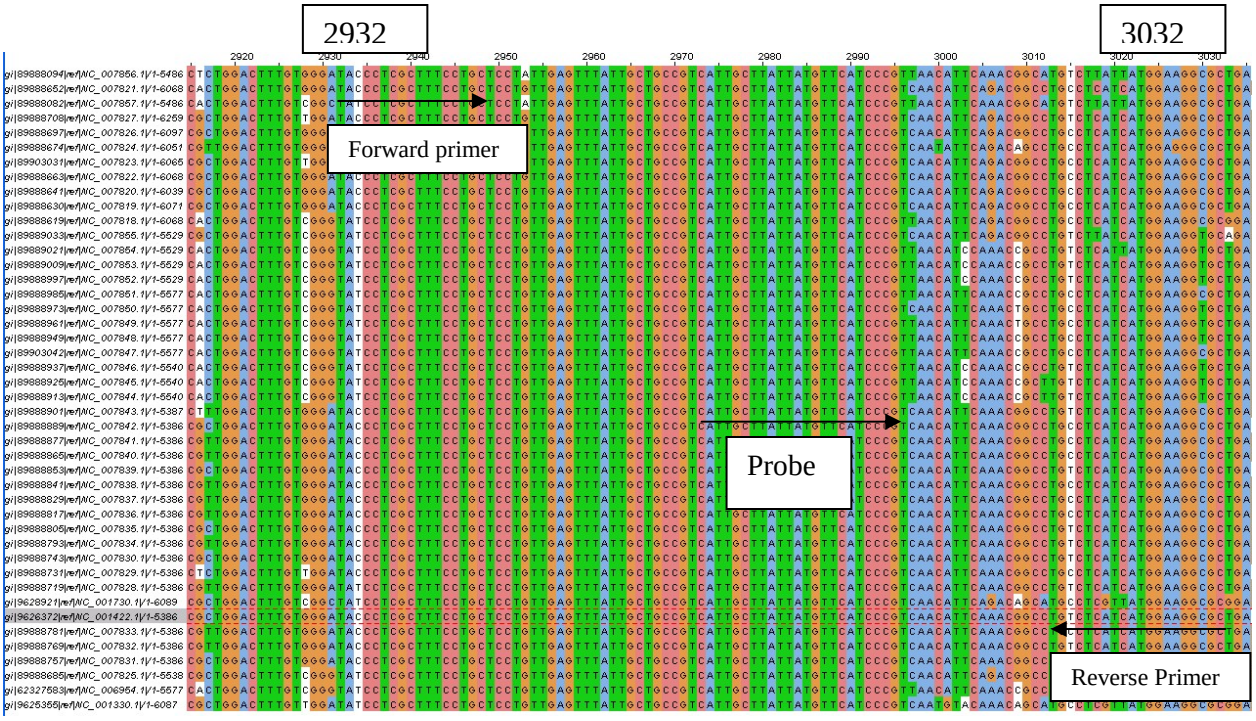
An antibody based rapid detection method for male-specific coliphage was developed and applied to environmental samples by Love and colleagues (Love *et al*., 2007). The latex particle immunoagglutination assay, which is widely used in clinical diagnostic microbiology (Hughes *et al*., 1984), was successfully applied to male-specific coliphage rapid detection in environmental samples. If production of polyclonal antisera against prototype strains of somatic coliphage is successful, it is prossible that rapid detection by CLAT assay can be developed and applied to somatic coliphages in environmental samples after initially culture enriching for a relatively short time.

The goal of this research is to develop new and rapid methods using somatic coliphages as feces-specific indicators to determine sources of microbial contamination in ambient waters used for drinking water supply, contact recreation and shell-fishing. The specific objectives are as follows: (i) develop real-time PCR assays targeting candidate somatic

coliphage groups; and (ii) develop somatic coliphage group specific antisera for rapid immunoassay screening and detection by a particle immunoagglutination method.

Materials and Methods

Target regions of the *Microviridae* family for real-time PCR assay A real-time PCR assay was developed for the *Microviridae* family. Previous work has shown members of this family to be abundant and persistent in fecally contaminated ambient waters. The *Microviridae* family is a single-stranded DNA group and the family having the smallest sized somatic coliphages. In the *Microviridae* family, there is a highly conserved region (shown in Figure 4.1), which served as the target region for family-specific amplification by real-time PCR methods. This conserved region in the *Microviridae* family was investigated as a part of capsid protein in this family. In order to find a conserved region in this genomic region for the detection of this family, multiple alignments were performed using the ClustalW2 program. Table 4.1 shows the selected primers and probe set for real-time PCR assay of the *Microviridae* family.



8. Figure 4.1 Conserved region of the *Microviridae* family in somatic coliphage after multiple alignments by the ClustalW2 program of all *Microviridae* family members at EBI website.

8. Table 4.1 PCR sequences in the *Microviridae* family for real-time PCR amplification.

	Sequences	Length	Location
Forward	5' TACCCTCGCTTTCCTGC 3'	17bp	
Reverse	5' GCGCCTTCCATGATGAG 3'	17bp	2932-3032
Probe	5' CATTGCTTATTATGTTCATCCCG 3'	23bp	

Optimization of TaqMan real-time PCR condition for the *Microviridae* family The primer sets and specific probe (Table 4.1) were tested and optimized in a TaqMan real-time PCR assay using PhiX174 as a positive control prototype strain of the *Microviridae* family. Amplification conditions were optimized by using a Smart Cycler real-time thermocycler (Cepheid, CA). The Primer set was tested by using SYBR Green for melting curve analysis. Following confirmation of the primer specificity, the probe was applied to the real-time PCR method. Two probe concentrations (0.25μM and 0.5μM) and several annealing temperatures (55-63°C) were tested to optimize real-time PCR conditions.

Real-time PCR method application Using the QIAamp viral mini kit (QIAGEN Inc.), viral DNA was extracted from 100ul volumes of archived somatic coliphage isolates that had been previously cultured. Then, 2ul of the resulting viral DNA was amplified by real-time PCR on a Smart Cycler, and the resulting Ct values were compared to a standard curve for quantification of virus concentration in samples.

Inhibition test for *Microviridae* family real-time PCR method To detect potential inhibitors of the real-time PCR method using the primers developed for the detection of the *Microviridae* family, tests were done using several water matrices spiked with a prototype strain, PhiX174. Surface water, seawater, tap water, and PCR-grade water were spiked with PhiX174, with PCR-grade water used as the reference sample for the inhibition test. After ten fold serial dilution of all different water matrices, DNA extraction and real-time PCR were performed. The Ct value for amplified PhiX174 DNA in each water matrix was compared to that of PCR-grade water to determine the effects of inhibitory substances on real-time PCR quantification. The test was performed two times.

Antibody preparation and production To screen environmental waters for the presence of somatic coliphages by CLAT assay, polyclonal antisera targeting individual somatic coliphage families were produced. Antisera were raised in rabbits in a commercial laboratory (Pacific Immunology corp.) using phage antigens in the form of inactivated, partially purified phage stocks. Briefly, each coliphage strain was propagated in an overnight broth culture of the bacterial host. The broth cultures were vigorously mixed with a 2:1 volume of fluorocarbon (Freon) for 2 min and then centrifuged (2,600g, 15 min. 4°C). Semi-purified

virus supernatant was retained. Each strain was further purified by filtering using a 0.22 μm pore size syringe filter, and then the filtrate was ultra-centrifuged at 35,000g for 4 hours. The virus pellet was re-suspended in 2ml of phosphate buffered saline (PBS). After concentration, the infectivity titer of each strain was between 10^8 and 10^9 virus plaque forming units (PFU) per ml. Protein concentration of purified stock was measured (Table 4.2). To measure the protein concentration of each purified phage stock, Bovine Serum Albumin (BSA) was used for generating a protein standard curve. The absorbance was measured at 595 nm by spectrophotometer, and the standard curve was used to estimate protein concentration in each virus stock. Due to low protein concentration after purification and ultracentrifugation of *Podoviridae* strain T7, *Podoviridae* strain N4 was used as antigen for antibody production.

9. Table 4.2 Positive somatic coliphage strains and protein concentrations of antigens prepared for antibody production

Family	Phage strains	*E. coli* host	Protein concentration (mg/ml)
Myoviridae	T4	*E. coli* B	0.5
Microviridae	PhiX174	*E. coli* C	0.4
Siphoviridae	T1	*E. coli* B	0.6
	Lambda	*E. coli* K12S Lederberg	0.8
Podoviridae	N4	*E. coli* W3350	1.0

Plaque reduction neutralization tests to titrate antisera potency A plaque reduction neutralization test was used to determine the antibody potency of an antiserum. Briefly, each antiserum was diluted serially 10-fold, and each antibody sample dilution was combined with a volume of phage containing approximately 100 PFU. The mixtures of antibody dilution and phage were kept at 37°C for 30 minutes to allow the antibodies to react with phages and neutralize their infectivity. After incubation, each combination of antiserum dilution and phage was added to a tube containing 3ml of molten agar. The appropriate *E. coli* host was

added and the mixture was poured into bottom agar plates. After overnight incubation, the plaques were counted in each antiserum dilution, including in an antiserum-negative control. The negative control for antiserum contained about 100 plaques. The neutralization potency titer of the antiserum was based on the greatest dilution that gave complete neutralization, as observed by the absence of plaques on the plates containing antibody.

Dot blot immunoassay of antisera tested against different somatic coliphage antigens

For dot blot immunoassay**,** a nitrocellulose membrane was pre-wetted in PBS and assembled into a sandwich using a standard apparatus (Bio-Dot Apparatus, Bio-Rad). Each well was washed with 50ul of PBS under vacuum. The rest of the procedure was performed under vacuum. Volumes of 50ul of diluted virus or *E. coli* host as test antigens were applied to each well and followed by another wash of 100ul with PBS. The membrane was removed from the apparatus, placed in a container, and 20ml of blocking solution (5% [W/V] milk in PBS) was applied on the membrane for overnight blocking at 4°C. The following day, the blocking solution was replaced with 10ml of diluted antibody or *E. coli* antigen in blocking solution. The membrane was incubated for 1hr at room temperature with slow shaking, followed by a wash step with 20~30ml of 0.05% Tween20/1X PBS three successive times. Secondary antibody (Peroxidase conjugated goat anti-rabbit IgG, Sigma) was applied for 1 hr at room temperature with slow shaking, and the wash step was repeated. For visualization, chemiluminescent peroxidase substrate (Chemiluminescent western blotting kit, Sigma) was used according to the manufacturer's instructions, and developed using an X-ray film cassette.

Reactivity of somatic coliphage antisera with different *E. coli* and somatic coliphage antigens by Enzyme-linked Immunosorbent Assay (ELISA) Volumes of 100uL of serially ten-fold diluted bacteria or virus antigen were applied to a 96 well plate (EIA/RIA plate, Costar) for 2hr at room temperature, and washed with 1X PBS. Then 300ul of blocking solution (5% [W/V] milk/PBS) was added in each well and incubated overnight at 4°C. On the following day, the blocking solution was discarded and 100ul of diluted antiserum was added and reacted for 1 hr at room temperature. The membrane was washed three times with 1X PBS/0.05% Tween20. A 100ul volume of secondary antibody (Peroxidase conjugate goat anti-rabbit IgG, Sigma) was applied to each well, reacted for 1hr at room temperature, and followed by washing three successive times with 1X PBS/0.05% Tween20. The membrane was developed by peroxidase substrate consisting of 9ml of 0.1M sodium acetate, pH 6.0, and 0.1mg of 3, 3', 5, 5' Tetramethylbenzidine, and 3ul of 30% Hydrogen peroxide. A 100ul volume of substrate was added to each well and 100ul of stop solution (1M sulfuric acid) was applied when the proper color was observed. The absorbance was recorded at 450nm by an Absorbance Microplate Reader (ELX 800, BioTek).

Western blots of somatic coliphage antisera against different antigens A 12% SDS-polyacrylamide gel and 5% stacking gel were prepared by following the protocol of ProtoGel Quick-Cast (National Diagnostics). Samples were prepared in equal volume with the 2X SDS Gel-loading buffer (100mM Tris-HCl pH 6.8, 200mM Dithiothreitol (DTT), 4% SDS (Electrophoresis grade), 0.2% Bromophenol blue, 20% Glycerol), and denatured at 95°C for 10 minutes. Then samples were separated at 100V for 5% stack gel and 150V for 12% separating gel in running buffer (1X Tris/Glycine/SDS, Bio-Rad) using a Mini-PROTEAN 3

cell (Bio-Rad) chamber. A protein ladder was run along with samples in the same gel. After gel electrophoresis, the separating gel was transferred to nitrocellulose membrane by western blotting apparatus (Mini-PROTEAN 3cell, Bio-Rad) kit with transfer buffer (39mM Glycine, 48mM Tris-Base, 0.037% SDS, 20% Methanol and pH 8.3) for 1hr at 100V. The transferred membrane was blocked with blocking solution (5% [W/V] milk/PBS) overnight at 4°C. On the following day, the membrane was incubated with diluted antiserum for 1hr at room temperature and then reacted with secondary antibody (Anti-rabbit IgG, Sigma) for 1hr at room temperature with three successive washing steps between each reaction. After the final wash step, the membrane was ready for developing by adding chemiluminescent peroxidase substrate (Chemiluminescent western blotting kit, Sigma), then placed in cassette for X-ray film development.

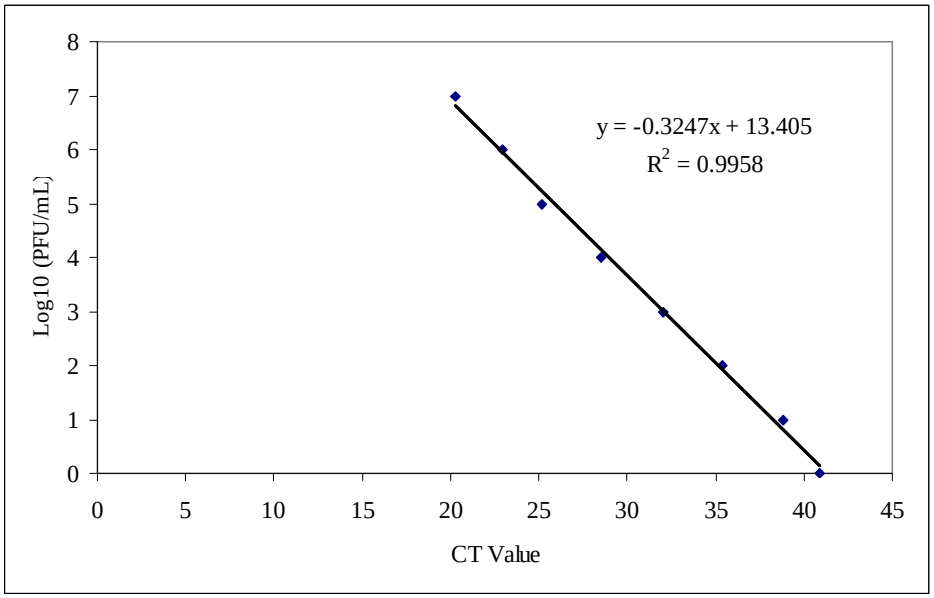
Culture, Latex Agglutination, and Typing (CLAT) CLAT assay was applied for rapid detection of the somatic coliphages by following the procedure of Love *et al*. (2007). Briefly, a 10% suspension of 0.29μm diameter polystyrene particles (OptiBind particles; Seradyn Inc. Indianapolis, IN) was diluted to 1% using phosphate buffered saline (PBS) buffer at pH 7.2 for highest binding efficiency (Love *et al*, 2007). Equal volumes of somatic coliphage antisera and PBS as negative control were added to the 1% polystyrene particle buffer solution. After mixing by pipetting for several seconds, the antibody and particle mixed solution were incubated for one hour at room temperature with mixing at 100 rpm (RKDYNAL, Dynal Biotech). After one hour incubation for adsorption of antiserum and particles, the sample was centrifuged for five minutes at 15,000 rpm in a micro-centrifuge. After the supernatant was decanted to remove unbound antibodies, the pellet was re-

suspended in 0.01% BSA-PBS buffer (pH 7.2) by pipetting. The CLAT detection solutions were stored at 4°C. Three somatic coliphage antiserum-labeled particle buffer solutions (anti-T1, anti-N4, and anti-T4) were prepared. After preparing the CLAT solution, 3µl volumes of the CLAT solution and an equal volume of somatic coliphage sample were spotted onto the circular black regions of agglutination cards (Pro-Lab Diagnostics; Austin, TX), mixed by pipetting and spread by toothpick for two seconds. The agglutination cards were rocked by hand for 30 - 60 seconds to promote agglutination of somatic coliphage and antibody-labeled CLAT detection particles. Clumping of the particles could be seen in positive samples. Negative samples appeared as milky solution with no clumping reaction. To optimize antisera concentration for detection in CLAT assay, two-fold serial dilutions (1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) of antisera T1, N4, T4 were applied to the CLAT assay. In addition, to determine detection limit of the CLAT assay, ten fold serial dilution of each somatic coliphage positive strain as antigen (prototype strains: T1, N4, T4, Lambda, and PhiX174) were applied to the CLAT assay.

Results

Standard curve and TaqMan real-time PCR conditions for the *Microviridae* family To optimize the probe concentration for real-time PCR, varying probe concentrations were used in the assay to detect a range of virus concentrations, and the lines generated by plotting Ct values against $\log_{10}$ virus concentration were compared. In optimization tests, a probe concentration of 0.5µM showed greater linearity in the graph of Ct value vs. $\log_{10}$ virus concentration compared to other probe concentrations tested. Therefore, 0.5µM of probe was used to produce the standard curve for subsequent experiments. Using this probe

concentration, PCR conditions were optimized. Optimized amplification conditions for real time PCR were: 95°C for 15min, and 45 cycles of 95°C for 10 sec, 61°C for 30sec, and 72°C for 15sec. The standard curve generated using PhiX174 as a prototype strain for the *Microviridae* family is shown in figure 4.2. The standard curve showed good linearity over 99% of r-square value for correlation analysis, and this standard curve was used to quantify the virus concentrations in samples archived from previous experiments.



9. Figure 4.2 Standard curve for real-time PCR method using optimized conditions for the *Microviridae* family of somatic coliphages. The positive control strain used for generating the standard curve was PhiX174.

Application of real-time PCR methods to the isolates of the time course survival study of somatic coliphages in OWASA sewage After optimizing real-time PCR using PhiX174, this method was applied to environmental somatic coliphage isolates collected over a time course survival experiment using effluent from a North Carolina wastewater treatment plant (OWASA). The isolates collected at each time point over the time course of the experiment were first identified by a family-specific conventional PCR method. Real-time PCR was then

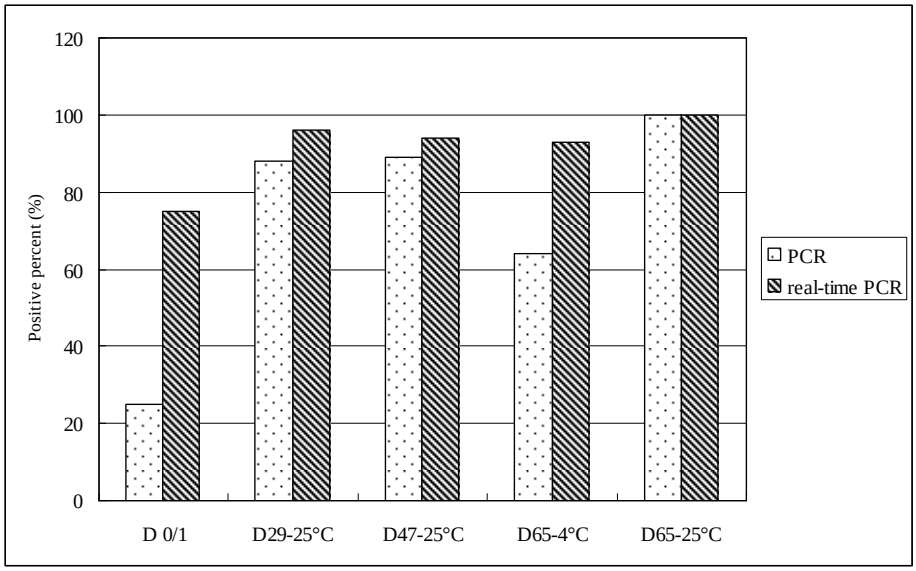
applied for their rapid detection. Table 4.3 shows the results of the real-time PCR method for somatic coliphage isolates from OWASA wastewater. The real-time PCR results were in agreement with the trend of family specific conventional PCR results. Over time, the *Microviridae* family was dominant in the somatic coliphage population. Ct values for all isolates were examined to determine if there were trends over time in the survival test. A decline in Ct values was seen in day 0. The average Ct value of Day 0/1 sample was 31.4, and. the average Ct values of day 29 (25°C), day 47 (25°C), day 65 (4°C), and day 65 (25°C) were 22.9, 24.1, 25.6, and 25.3, respectively.

10. Table 4.3 Presence of the *Microviridae* family among somatic coliphage isolates from OWASA wastewater treatment plant as a function of sample incubation time as detected by real-time PCR

Days	0-1	29(25°C)	47(25°C)	65(4°C)	65(25°C)
No. *Microviridae* Positives/Total No. Isolates	36/48	25/26	34/36	42/47	3/3
Percent (%) Positive	75	96	94	93	100

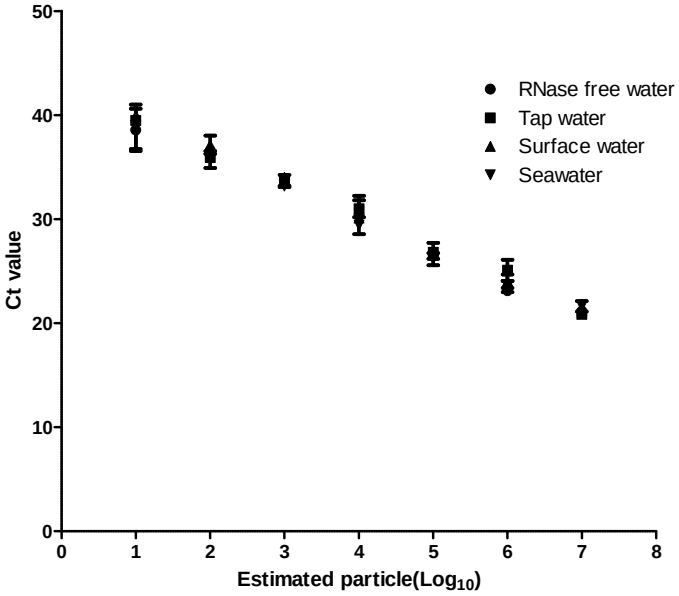
Comparison of real-time PCR and conventional PCR methods to detect the *Microviridae* family in somatic coliphage isolates from OWASA All isolates from the sewage effluent time course survival test were examined by real-time PCR and conventional PCR. Results from both methods are shown in figure 4.3. Real-time PCR resulted in more positive samples than conventional PCR. In total, real time PCR methods detected *Microviridae* in 20 samples that were negative by conventional PCR. Therefore, compared to the conventional PCR, the real-time PCR method detected *Microviridae* in a greater number of archived isolates. This result suggests that the real-time PCR method is more sensitive than the conventional PCR method for somatic coliphage detection. The detection limit of

conventional PCR was measured by using a serial dilution of PhiX174, and a detection limit of 300 PFU was found (Table 3.5). However, in the real-time PCR, the detection limit was 0.3PFU. The sensitivity of real-time PCR could contribute to the observed differences in the number of positive samples identified as belonging to the *Microviridae* family among the isolates from North Carolina sewage effluent.



10. Figure 4.3 Percent positivity for the *Microviridae* family among isolates from OWASA by the real-time PCR method and Conventional PCR using *Microviridae* family specific primers. Dot bar: by conventional PCR, diagonal line bar: by real-time PCR

Inhibition test of real-time PCR in different water matrices After applying real-time PCR to different water matrices, the Ct value of each water matrix was compared. The inhibition test was performed twice, and the result is shown in figure 4.4. The highest estimated phage concentration of the test was 10^7 PFU, and the lowest estimated concentration was 10^1 PFU. There were no significant differences between the four water types (One way ANOVA, p=0.9968) for quantitative real-time PCR Ct values of *Microviridae* representative PhiX174.



11. Figure 4.4 Results of inhibition test of real-time PCR methods by using different matrixes after spiking PhiX174 as a positive control in the *Microviridae* family: Error bar shows standard deviation

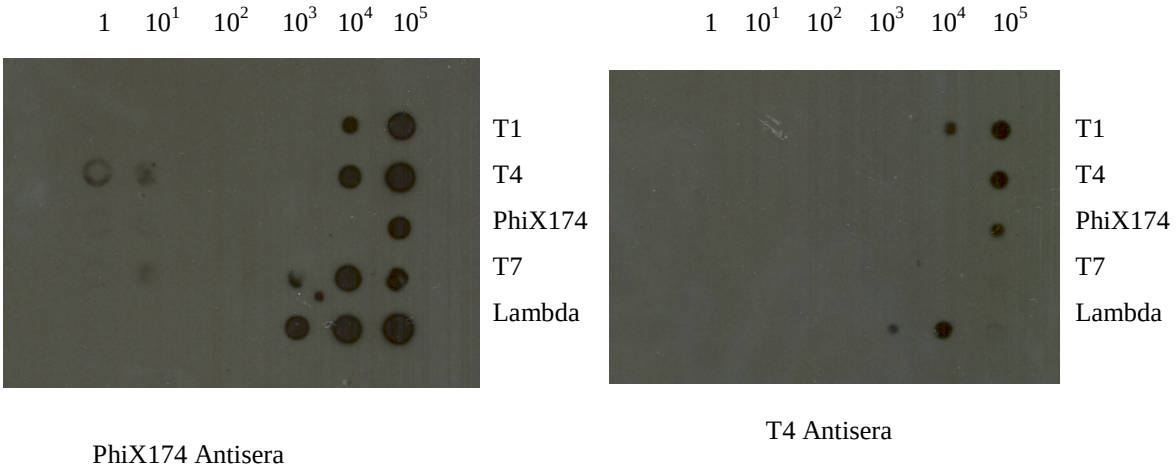
Plaque reduction neutralization tests to titer antisera The plaque reduction neutralization test was used to determine the potency of produced antiserum. There were 4 productions and one pre-bleed (pre-immune serum collection) in total for each antiserum. All batches were tested by plaque reduction neutralization. Table 4.4 shows the measured titer of each antiserum. There is no change of titer from the first produced antiserum to the final produced antiserum. Antiserum neutralization titer ranged from a low of 10^0 to a high of 10^3, which are low neutralization titers compared to the antisera previously produced for F+ coliphage serotypes (Love and Sobsey, 2007).

11. Table 4.4 Results of antisera titration by plaque reduction neutralization antisera assay of five strains in four families of somatic coliphages.

Family	Antiserum	Titer against each phage
Myoviridae	T4	10^2
Microviridae	PhiX174	10^3

Siphoviridae	T1	10^0
	Lambda	10^0
Podoviridae	N4	10^2

Dot blot immunoassay of antisera tested against different somatic coliphage and *E. coli* host cell antigens Both antiserum-PhiX174 and antiserum-T4 were analyzed by dot blot immunoassay to measure specificity with various dilutions of somatic coliphages T1, T4, T7, PhiX174, and Lambda. The result is shown in figure 4.5. Most of the somatic coliphage strains had a positive reaction against PhiX174 and T4 antisera. Moreover, there is evidence that they reacted positively against their *E. coli* host antigens. Both antisera tested also had a cross reaction between coliphages. To further investigate the specificity of all antisera and their hosts, Enzyme-linked Immunosorbent Assay (ELISA) was applied.



12. Figure 4.5 Results of dot blot assay of antisera PhiX174 and T4 against somatic coliphage antigens T1, T4, PhiX174, T7, and Lambda with serial dilution. For antisera PhiX174 and T4, 10^{-3} and 10^{-2} dilution of antisera PhiX174 and T4 were used for dot blot immunoassay

Reactivity of somatic coliphage antisera with different *E. coli* and somatic coliphage antigens by Enzyme-linked Immunosorbent Assay (ELISA) All *E. coli* host strains and

phages were tested for cross-reaction by the ELISA method. Anti-PhiX174 reacted with *E. coli* host bacteria antigen, but not with PhiX174 itself. Anti-T4 also reacted with the *E. coli* host bacteria antigen, as well as with the T4 and N4 strains. Anti-T1 also reacted with both *E. coli* host bacteria antigens and members of the same somatic coliphage family (T1, Lambda, and HK97 are all *Siphoviridae* family). Anti- Lambda reacted with all *E. coli* hosts and somatic coliphages. Therefore, there was no specificity of anti-Lambda antiserum. Anti-N4 did not react with the host bacteria but only with the N4 strain itself. Therefore, based on these ELISA results, antiserum-N4 and T1 were applied to CLAT assay directly for N4 and *Siphoviridae* family detection, respectively (Table 4.5 and 4.6).

12. Table 4.5 Results of ELISA to test specificity and sensitivity of five antisera (PhiX174, T4, T1, Lambda, and N4) against available *E. coli* host strains and somatic coliphages. The number in the parenthesis showed the dilution factor of antisera after optimization for ELISA.

Tested Ag	Anti-PhiX174(-3)	Anti-T4(-2)	Anti-T1(-3)	Anti-Lambda(-3)	Anti-N4(-3)
E. coli K12S Lederberg	-	-	-	-	-
E. coli B	++	+++	+++	-	-
E. coli CN-13	-	-	-	-	-
E. coli C	++	-	-	-	-
E. coli W3350	-	-	-	-	-
E. coli MUL70.1	-	+++	-	-	+++
E. coli 40	-	-	-	-	-
E. coli Y mel	-	-	-	-	-
PhiX 174	-	-	-	-	-
T 1	-	-	++	-	-
T 4	-	++	-	-	-
T 7	-	-	-	-	-
Lambda	+++	-	++	-	-
HK97	+++	-	++	-	-
N4	-	+++	-	-	+++
MS2	-	-	-	-	-

++: Showed reaction at -2 dilution level of antigen

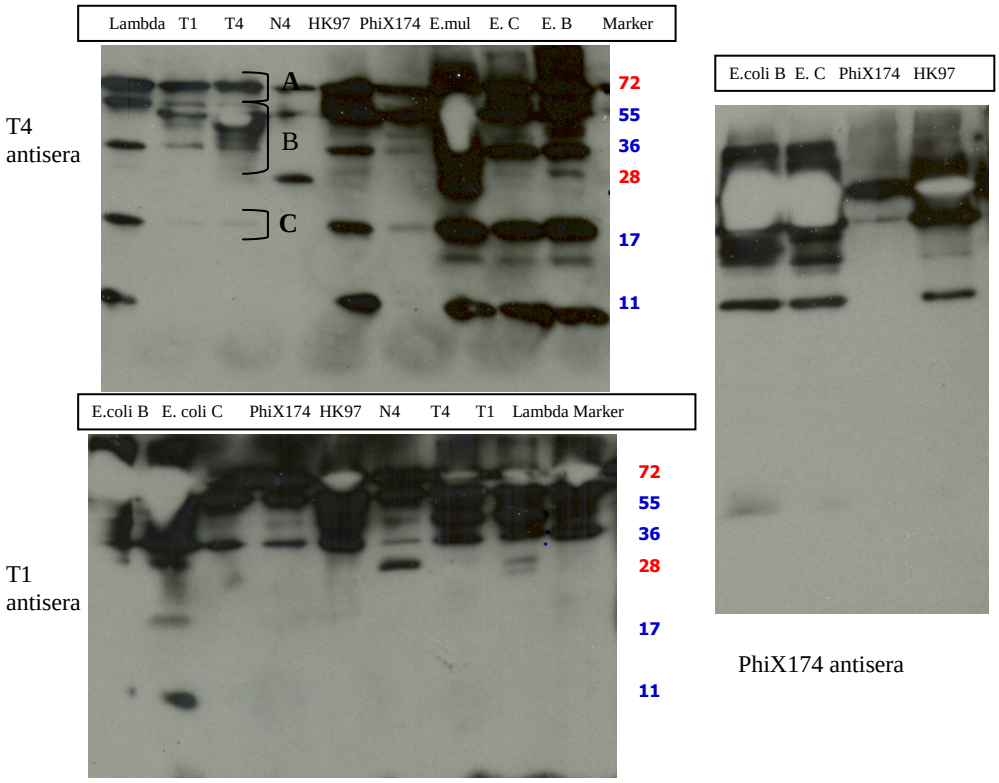
+++: Showed reaction at -3 dilution level of antigen

13. Table 4.6 Summary of ELISA test for five antisera against *E. coli* host and somatic coliphage antigens

Antiserum	Titer for Ag for Infectivity	Interaction test by ELISA		
		E. coli	Phage	Tested dilution
PhiX174	$1:10^3$	B C	Lambda HK97	in -3 dilution
T4	$1:10^2$	B MUL-B70.1	N4 T4	in -2 dilution
T1	$1:10^0$	B	T1 Lambda HK97	in -3 dilution
Lambda	$1:10^0$	All	all	in -3 dilution
N4	$1:10^2$	MUL-B70.1	N4	in -3 dilution

Western blots of somatic coliphage antisera against different antigens Antisera T1, T4 and PhiX174 showed cross reaction with their hosts. To further characterize antisera, anti-T1, T4, and PhiX174 were tested by Western blot analysis (Figure 4.6). Antiserum PhiX174 was tested against *E. coli* B, *E. coli* C, PhiX174, and HK97. The western blot result of antiserum PhiX174 showed no specific protein band from phage or host. Therefore, it was not possible to detect a mono-specific antibody against PhiX-174 in the PhiX174 polyclonal antiserum. The same result was also observed in antiserum T1 tested against *E. coli* B, *E. coli* C, PhiX174, N4, T1, T4, HK97 and Lambda. The similar protein patterns from reaction with T1 antiserum among all the hosts and phages indicated little evidence for mono-specific antibody from anti-T1 polyclonal antisera. For the antiserum T4, the strains showing any positive reaction in ELISA were investigated by western blot, specifically to distinguish N4 strain from T4 strain in antiserum T4. T4 viral proteins were separated by 12% SDS-PAGE, transferred on nitrocellulose membrane by the western blot method, and probed with T4 antiserum. Three portions of antibodies, marked as A, B and C on Fig 4.6, were recovered from the membrane with 100mM glycine pH 2 and neutralized by 1M Tris pH 8.0. Three isolated antibodies (A, B and C) from T4 antiserum were tested by ELISA for strain

specificity. However, the ELISA result confirmed the previous experimental results showing that there is a cross reaction between T4 and N4 strains by antiserum T4.



13. Figure 4.6 Results of western blot for antisera T4, T1, and PhiX174 for further protein characterization of each antiserum. Protein marker is shown in the middle of this figure. All strains previously showing cross-reactivity were tested and are presented in the box above the figure. For antiserum T4, the nitrocellulose membrane was cut with A, B, and C, and tested for strain specificity by ELISA.

Immunoassay for somatic coliphage screening and typing Antisera against somatic coliphage strains were produced to develop a rapid immunoassay for somatic coliphage screening and typing based on particle immunoagglutination assay, called CLAT assay. Antibody quality, based on taxonomic group specificity and titer, was determined using infectivity neutralization tests, dot blot immunoassay, ELISA, and Western blots. After confirming which antibodies were specific for each strain or family, CLAT assay was applied

for the use these antibodies in particle immuno-agglutination (CLAT) assays after the method of Love *et al*. (2007). The immunoassay method was used for identification of somatic coliphages isolates from sewage effluent, similar to the use of the method that has already been developed for F+ coliphages. Antiserum N4 labeled particle solution was used to investigate N4 strain of the *Podoviridae* family, and Antiserum T4 labeled particle solution also was used for finding T4 strain of the *Myoviridae* family among somatic coliphage isolates. Even though antiserum-T4 shows weak cross-reaction with N4, there is specificity of antiserum-N4 for detecting N4 strain. Therefore, antiserum T4 was applied to CLAT assay to detect T4-like strains among somatic coliphage isolates. Also, antiserum T1-labeled particle solution was used for exploring somatic coliphage isolates for the presence of members of the *Siphoviridae* family among them.

Optimal dilution of antiserum-labeled agglutination particles To investigate the optimal dilution of antiserum-labeled particles and the concentration of antisera in the CLAT assay, two-fold dilution series of each antisera were applied to the CLAT assay. The optimal concentration of each antiserum is shown in Table 4.7. The 1:64 dilutions of N4, T1, and T4 were used for the CLAT assay to determine if there was cross-reaction with other antigens. These optimal concentrations were applied to test the other somatic coliphage strains for exploring cross-reactions. As shown in Table 4.7, the N4-labeled particle solution showed strain specificity, and T1-labeled particle solution showed *Siphoviridae* family specificity. T4-labeled particle CLAT solution showed T4 strain specificity with a weak cross-reaction with the N4 strain. These results support the ELISA assay results of the previous experiments.

14. Table 4.7 Optimized dilution conditions of CLAT assay with antiserum-coated particles and each somatic coliphage and the results of cross-reactivity among non-target somatic coliphage strains.

Somatic coliphage strains and negative control	Somatic coliphage antiserum labeled particles and dilutions[1]		
	N4 (1:64)	T1 (1:64)	T4 (1:64)
N4	+	-	+(w)[2]
T1[3]	-	+	-
Lambda[3]	-	+	-
HK97[3]	-	+	-
T4	-	-	+
PhiX174	-	-	-
Pre-bleed of each antisera	-	-	-

[1]: Antisera dilutions of 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 were used to determine optimal antibody dilutions in a checkerboard titration. The selected antibody dilutions were used to examine cross reactivity with each non-target somatic coliphage strain.
[2]: Weak positive after 60 seconds
[3]: *Siphoviridae* family

Lower detection limit of CLAT assay The lower detection limits of the CLAT assay are shown in Table 4.8. The somatic coliphage prototype strains were cultured by SAL (EPA 1602, 2001). The somatic coliphages were assayed by ten folded serial dilution with appropriate antiserum-labeled particle solution. T1-antiserum labeled particles were tested with T1, Lambda, and HK97 as a family-specific antiserum. The lower detection limit of different target somatic coliphages ranged from 2×10^3 to 3×10^6 PFU/10ul (Table 4.8).

15. Table 4.8 Lower detection limits of somatic coliphage positive strains using antiserum labeled polystyrene particles

Somatic coliphage antiserum-labeled particles (antiserum dilution)	N4 (1:64)		T1[1] (1:64)		T4 (1:64)
Somatic coliphage prototype strains	N4	T1	Lambda	HK97	T4
Lower detection Limit (PFU[2]/10ul)	2×10^6	2×10^5	4×10^3	8×10^3	3×10^6

[1]: T1 antiserum-labeled particles were tested by all *Siphoviridae* strains as antigens: T1, Lambda, and HK97
[2]: PFU: Plaque Forming Unit

Discussion

Previous studies have focused on developing methods to rapidly detect fecal indicator microorganisms including *E. coli*, fecal coliforms, and male-specific coliphages. Multiple methodological approaches for rapid detection methods have been investigated for application to environmental water samples such as enzyme-medated substrate based assay, ATP based assay, molecular assay, and antiserum based assay (Bushon *et al*., 2008; Stanek *et al*., 2001; Stender *et al*., 2001; Yong *et al*., 2006). The aim of rapid detection methods is to detect fecal contamination in water before drinking water reaches the consumer, bathers get exposed to contaminated recreational water or shellfish are harvested from contaminated growing waters, as examples. Culture-based detection methods require incubation times of at least 18-24 hours, with results coming only after water has been delivered to the consumer, recreational bathers have been exposed or shellfish harvested and delivered to consumers. Somatic coliphages are promising potential indicators for the presence of enteric viruses in water, and rapid detection methods are needed if somatic coliphages are to be used as indicators for timely protection of public health and for timely security of safe drinking water.

In this study, two approaches were taken for developing rapid detection methods for somatic coliphage taxonomic groups as candidate viral indicators. Real-time PCR was developed and evaluated for the detection of the *Microviridae* family as a possible fecal indicator virus family. In addition to the molecular based rapid detection method approach, an antibody based rapid detection method (CLAT, Love *et al*., 2007) was evaluated for each family of somatic coliphage by producing polyclonal antiserum for representative strains in each somatic coliphage family.

Real-time PCR was developed to detect the *Microviridae* family based on their highly conserved regions of the viral genome. Instead of using the whole somatic coliphage group

as an indicator, the *Microviridae* family was chosen as an indicator that could potentially be more specific for the presence of human enteric viruses, due to their persistence and abundance in water. After multiple alignments of the viruses in this family, a highly conserved region was identified for real-time PCR amplification. The standard curve generated using the conserved genomic region in this family showed enough linearity and high Ct values to detect the *Microviridae* family in environmental samples. Isolates from North Carolina sewage effluent were tested with this developed real-time PCR method to detect the *Microviridae* family. In total 160 isolates were examined, and 140 (87.5%) were identified as members of the *Microviridae* family. The proportion of positive *Microviridae* at each sampling point increased over time in a time course survival test. After 29 days of the coliphage survival test, over 90% of isolates were identified as belonging to the *Microviridae* family using this method.

To determine possible inhibition in the real-time PCR method when applied to different water matrices, the developed real-time PCR was applied to RNase free water, drinking water, seawater, and surface water. The differences of Ct values in each water matrix including the seawater sample were not significant when they were statistically compared. Therefore, the real-time PCR method in this study could be applied to a range of environmental water samples, including drinking water, surface water, and seawater.

An antibody-based detection method was considered as another approach to rapid detection of somatic coliphages. Previously, a latex (particle) immunoagglutination method used in clinical microbiology had been successfully applied in a rapid and effective screening method to detect and characterize F+ coliphages in environmental waters, after initial

enrichment culture, to provide a simple and rapid detection tool for F+ coliphages (Culture, Latex, Agglutination &Typing (CLAT)) (Love *et al.*, 2007).

To apply those possible methods to detect somatic coliphages, antiserum for each representative strain of each somatic coliphage family was produced and evaluated for its sensitivity and specificity. The titers of antisera were measured and the antisera were used for sensitivity and specificity assays by plaque reduction neutralization tests. After serial assay by dot blot immunoassay, ELISA, and western blot for testing specificity of polyclonal antisera of each somatic c coliphage strain, antisera T1, T4, and N4 showed effective strain or family level specificity. However, antisera PhiX174 and Lambda showed no sensitivity and specificity at strain or family level by serial immunoassays. Also, most of the developed antisera showed cross reactivity with their *E. coli* host but not with *E. coli* CN-13. The somatic coliphage isolates from sewage were grown on *E. coli* CN-13 as their hosts, so the antisera were used to directly detect strains or families. However, another purification process should be considered to eliminate the cross reaction with other host *E. coli* strains. Specifically, antiserum T1 showed specificity for the *Siphoviridae* family such as T1, Lambda, and HK97 in this study. Although all of the strains in the *Siphoviridae* family were not tested in this assay, antiserum T1 showed sufficient specificity for *Siphoviridae* strains tested in this assay. This result suggests family specificity of antiserum T1 for CLAT assay. In addition, T4 and N4 also showed strain specificity in serial immunoassays. Therefore, both antiserum T4 and N4 as a strain level assay reagents and antiserum T1 as a family level assay reagent were used for the detection of these somatic coliphages as environmental isolates by CLAT assay.

Before applying produced antisera to environmental isolates by CLAT assay, the optimal conditions for this assay were investigated using available positive stains. Antiserum T1, T4 and N4 were optimized for the degree of dilution to use for labeling agglutination test particles. Each antiserum absorbed onto particles at the optimum dilution was applied to CLAT assay and examined for cross-reaction with the other strains. As shown in table 4.7, the results were similar to those by ELISA. The optimized test condition of each antiserum was applied to environmental isolates to detect T4-like strains, N4-like strains, and *Siphoviridae* family members among the somatic coliphage isolates from water. Also, the detection limit of each antiserum was examined by assaying ten fold serial dilutions of each prototype strain as a positive control. The lower detection limit of CLAT assay for T1, T4, and N4 was found to be in the range of 10^3 to 10^6 PFU per 10ul (Table 4.8). These CLAT lower detection limits to detect T4, N4, and *Siphoviridae* family of somatic coliphage were sufficiently low for their detection after a culture enrichment step was applied to amplify the numbers of somatic coliphages in samples of environmental waters.

The CLAT assay, previously developed for F+ coliphages (Love *et al*., 2007), was successfully applied to somatic coliphages in this study even though effective reagents for all of the somatic coliphage families were not developed with success due to lake of specificity and sensitivity of produced polyclonal antisera. However, the CLAT assay is a simple, rapid, and inexpensive method for coliphage detection if antisera against somatic coliphage strains show sufficient sensitivity and specificity. Also, there is room for improvement of the CLAT assay in several ways, such as exploring more effective buffer solution instead of PBS (pH 7.2) and use of more specialized beads with greater sensitivity. Even though the developed CLAT assay for somatic coliphage was not able to detect all somatic coliphage families, this

method provides more opportunity to investigate the distribution of members of the somatic coliphage population in environmental waters.

The rapid and simple detection of somatic coliphage by nucleic acid based or antibody based methods could provide rapid and more specific water quality assessments if individual somatic coliphage families, subgroups or strains otherwise serve as a reliable viral indicator. The rapid detection methods for individual somatic coliphage families and their sub-groups make it possible to investigate the potential of these somatic coliphages to be a viral indicator in environmental waters. Further study is needed to investigate the correlation of enteric viruses and individual somatic coliphages or coliphage groups (subgroups or families) in water. The developed rapid detection methods for somatic coliphage families can be applied to identify their relationships and distribution in waters. Further development and validation of rapid detection methods for candidate viral indicators could provide more rapid water quality monitoring by effective detection of those individual families, subgroups or strains of somatic coliphages that are indicative of the presence of sewage and the possible presence of enteric viruses.

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CHAPTER 5 Application of rapid detection methods for somatic coliphage families in environmental samples

Abstract

Methods for the rapid detection of of reliable viral indicators that predict viral contamination in water are essential to protect public health. An individual somatic coliphage family, the *Microviridae*, could be a reliable viral indicator based on its relative persistence and abundance in environmental water. The aim of this research is to apply two rapid methods, real-time PCR and CLAT, for detecting members of specific somatic coliphage families as candidate fecal viral indicator viruses. Primary sewage in different regions, seawater, and groundwater samples were investigated using the rapid detection methods. Rapid detection of the *Microviridae* family by a real-time PCR method was successfully applied to a range of environmental water samples including primary sewage effluent in different geographic regions (Chapel Hill, NC; Chungbuk, South Korea), seawater, and groundwater. Members of the *Microviridae* family were persistent and abundant in sewage of both of regions, although the genetic diversity in the *Microviridae* family differs between regions. Rapid enrichment culture in seawater was optimized by different media and culture time for rapid somatic coliphage detection. A 7-hour enrichment culture was not significantly different from overnight enrichment culture for somatic coliphage detection in seawater and groundwater. Also, an antibody-based rapid detection method, CLAT (Culture,

Latex Agglutination, and Typing), for the *Siphoviridae* family, N4 (*Podoviridae*), and T4 (*Myoviridae*), was successfully applied to somatic coliphage isolates, although there is a need for improvement in method sensitivity. The development of rapid methods for detection of an individual somatic coliphage family and their successful application to environmental samples provides a new analytical tool to investigate individual somatic coliphages as reliable indicator viruses.

Introduction

Rapid detection methods for viral indicators in environmental waters are necessary for rapid assessment of water quality for timely response to public health threats. The importance of rapid detection of indicator microorganisms to estimate viral contamination in vulnerable waters has been suggested by many previous researchers (Kir *et al*., 2007, Love *et al*, 2007, Sim *et al*., 1995, Stanek *et al*., 2000, and Wentsel *et al*., 1981). To measure water quality in a timely manner, rapid detection of indicator microbes of interest is a potentially effective approach for public health protection and for regulatory purposes. There are promising new approaches to rapid viral detection of fecal indicator viruses in contaminated water systems (Hughes *et al*., 1984; Love *et al*., 2007, Fiksdal *et al*., 2008). Somatic coliphages are promising candidate indicators of enteric viruses in water, based on their persistence and abundance in environmental waters over time (Lee, unpublished data). Rapid detection methods for somatic coliphage have been further developed to distinguish each family of using two different approaches: real-time PCR and an antibody-based Culture, Latex Agglutination, and Typing (CLAT) assay.

The ecology of somatic coliphages in environmental waters is not thoroughly understood. Early studies suggested little seasonal variation of somatic coliphage densities in raw sewage (Kott *et al*., 1974), whereas in contrast, more recent studies found significant seasonal variation in the proportions in F+DNA and RNA coliphages (Cole *et al*., 2003). Newly developed rapid detection methods for somatic coliphage families need to be applied to different types of environmental waters to understand the ecology and survival of somatic coliphages in diverse aquatic environments, including groundwater and seawater. In addition, rapid detection methods can facilitate the understanding of the ecology of coliphages in human sewage as an important potential source of fecal contamination in environmental waters. The goal of this research is to apply two rapid methods, real-time PCR and CLAT, for detecting members of specific somatic coliphage families as feces-specific indicators of human enteric viruses in water. Detection methods were applied to a variety of waters, including primary sewage effluent, seawater for recreation and shell-fishing, and groundwater sources used for drinking water.

To investigate possible regional differences in sewage, primary sewage effluents from wastewater treatment plants in the United States and South Korea were examined. Sewage contains a variety of physical, chemical and microbiological contaminants that may differ with the life styles of populations contributing to the waste stream and other factors. Therefore, investigation of samples from different regions can give a better understanding of somatic coliphage ecology and their distribution in human sewage. To apply rapid methods to seawater used for primary contact recreation, samples from Malibu Beach in California were analyzed by real-time PCR. The application of real-time PCR methods to seawater is important to estimate and predict contamination of recreational water and to examine the

effects of PCR inhibitors in seawater on method performance. Groundwater serves as a source of drinking water for many regions, and is consumed often without treatment. Therefore, the successful application of rapid coliphage detection methods for the estimation of groundwater virological quality in a short time is important to protect public health. Newly developed rapid molecular and immunological detection methods were applied to these different samples to determine the effectiveness of these methods for the detection of somatic coliphages in different environmental media.

Materials and Methods

Sample Sources Primary sewage effluent sources were Orange [County] Water and Sewer Authority (OWASA), the wastewater treatment plant serving Chapel Hill, NC USA and K-water located in Chungbuk in South Korea. The primary sewage effluent collected in South Korea (K-water) was sent to our laboratory by air shipping. Total times for collecting and shipping of primary sewage effluent sample from South Korea took about ten days. A total of about 150 samples of sea water, collected at Malibu Beach (CA), were shipped to our laboratory for analysis from May to September, 2009. A total of 16 groundwater samples were collected near Malibu beach (CA) and shipped to our laboratory for analysis in July, 2009.

Enrichment culture assay for coliphage Somatic coliphages of seawater and groundwater samples were examined by the enrichment liquid culture method using three-volume MPN quantification in triplicate with volumes of 30, 3, and 0.3ml (EPA 1601, 2001 and Sobsey *et al.*, 2004). Log phase *E. coli* CN-13 was used as a host for somatic coliphage detection. After

a media optimization study, Luria Broth (LB) without NaCl was used for enrichment in place of the standard Tryptic Soy Broth (TSB). The spot plate method was used for the detection of enriched somatic coliphage both after 7 hour incubation for rapid detection at 36°C and after overnight enrichment at 36°C. Before spot plating, sample aliquots were centrifuged at 13000 rpm for 2 minutes to remove host cells and cell debris. A 10ul volume of sample was spotted in each assay. The results were recorded as positive or negative depending on the presence or absence of a clear zone of lysis within each spot. The concentration of somatic coliphage was computed as MPN/100ml.

Optimization of enrichment culture conditions for rapid detection in seawater To find the optimal enrichment culture detection conditions for somatic coliphage in seawater, different bacterial culture media were evaluated. PhiX174 was used as a test organism for optimization. Viral stock was diluted to 10-15 PFU/volume and then this volume was spiked into prepared water sample of 100ml volume. Test media were Tryptic Soy Broth (TSB) and Luria Broth (LB) without NaCl. Dilution of seawater with reagent water to lower the NaCl concentration in the assay was also evaluated. In addition, results for enrichment times of 1, 3, 5, and 7 hours post-inoculation were compared to results from standard overnight culture. Triplicate samples were assayed for each optimization factor.

Culture, Latex Agglutination, and Typing (CLAT) Assay Polyclonal antisera against somatic coliphages T4, N4, and T1 raised in rabbits in a commercial laboratory (Pacific Immunology Corp.) were used in the CLAT assay for somatic coliphage isolates. The CLAT reagents of each antiserum were subjected to optimization studies in an attempt to best

identify somatic coliphages by this method. Using antisera with sufficient specificity and sensitivity as confirmed by ELISA assay, the optimized CLAT assay was applied to a total of 20 somatic coliphages isolated from OWASA sewage. A CLAT reagent suspension consisting of particles labeled with Antiserum N4 was used to investigate the presence of N4-like strain of the *Podoviridae* family among the isolates. Likewise, a particle suspension labeled with antiserum T1 was used for determining the presence of members of the *Siphoviridae* family among the isolates. Antiserum T4-labeled particle solution also was used for finding T4 strain of the *Myoviridae* family in the isolates. A 1:64 dilution of each antiserum was used. After preparing the CLAT solution, 3µl volumes of the each CLAT solution of antiserum and an equal volume of somatic coliphage isolates were spotted onto black agglutination cards (Pro-Lab Diagnostics; Austin, TX), mixed by pipetting and spread by toothpick for two seconds. Cards were rocked by hand for 30 - 60 seconds to promote agglutination of coliphage and antibody-labeled CLAT detection particles. Clumping of the particles could be seen in positive samples. Negative samples appeared as a milky solution with no clumping reaction.

Sample preparation and processing for survival test of somatic coliphages in K-water sample Samples of primary sewage effluent from South Korea were held at two temperatures, 25°C and 4°C, for 34 days. Aliquots were taken from the samples at the beginning of the experiment, and at 10, 20 and 34 days. Somatic coliphages were quantified and isolated from the original samples at each time point, and were enumerated using single agar layer plaque assay (SAL, EPA 1602, 2001). A representative number of somatic coliphage plaques (30-50) were randomly picked from SAL plates at each time point.

Plaques were picked by using the tip of a micropipettor (20-200ul capacity) set at the 100ul volume, suspended in 100ul of TSB and assayed using a spot plate technique, with individual plaques in each spot counted to determine sample titer. Individual plaques from each spot were picked, re-suspended, and re-enriched for 24 hours in TSB using host bacterium *E. coli* CN-13. After incubation, the enriched phage isolates were filtered to remove cell debris using 0.45 um pore size filters, and frozen at -80°C for further characterization.

Group-specific conventional PCR and real-time PCR methods for detection of the *Microviridae* family Optimized group-specific conventional PCR and real-time PCR methods were applied to detect the *Microviridae* family. Viral DNA was extracted from archived isolates by using the QIAamp viral mini kit (QIAGEN Inc.). After real-time PCR in a Smart Cycler (Cepheid, CA), the Ct value of each isolate was examined to estimate the concentration. Also, group-specific conventional PCR by using specific *Microviridae* primers was used for detecting the *Microviridae* family in the collected isolates.

Sequencing of positive samples and phylogenetic analysis For further characterization of somatic coliphage environmental isolates, PCR products were purified (QIAquick PCR Purification kit; QIAGEN Inc.) and sequenced at the Genome Analysis Facility (University of North Carolina, Chapel Hill). Multiple sequence alignments and clustering of the sequenced isolates along with *Microviridae* full genome sequences from NCBI website (http://www.ncbi.nlm.nih.gov/) were conducted with the ClustalW2 program at the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/). Phylogenetic analysis was conducted using the Neighbor-Joining method (Saitou *et al.*, 1987). The percentage of

replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is displayed next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method as the substitution model (Kimura, 1980**)**. Phylogenetic analyses were conducted in MEGA4 (version 4, Tamura *et al*., 2007).

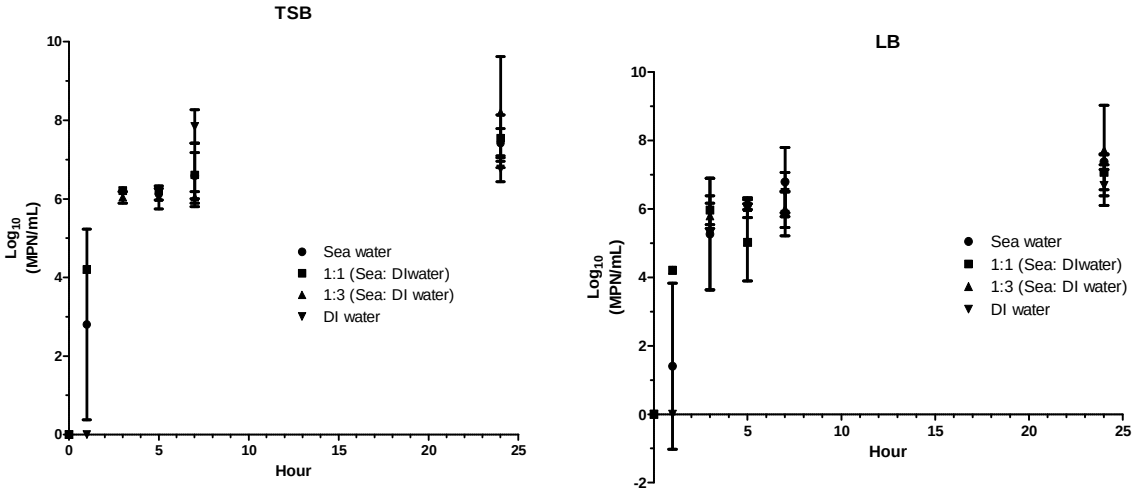
Results

Application of CLAT assay to environmental isolates The somatic coliphage isolates from sewage effluent from OWASA were analyzed using the optimized CLAT assay to determine their strain or family in the somatic coliphage population. Using antisera with sufficient specificity and sensitivity, CLAT assay was applied to a total of 20 somatic coliphages isolated from North Carolina municipal sewage. Antiserum N4-labeled particle solution was used to investigate the presence N4 reactive strains of the *Podoviridae* family among the isolates, and antiserum T1-labeled particle solution was used for exploring the presence of *Siphoviridae* family members among the isolates. Antiserum T4-labeled particle solution also was used for finding T4-like strains of the *Myoviridae* family among the isolates. The results of these analyses were that none of the 20 somatic coliphage isolates could be identified to the family level using these antisera.

Optimized condition for incubation time and culture media Using PhiX174 as a test organism, the culture conditions for the enrichment step of the somatic coliphage assay were optimized to decrease the inhibition of phage growth caused by constituents in seawater samples. Media type, enrichment time and sample dilution factor were varied and their effect on the rate of increase in viral titer was observed. Figure 5.1 shows the results of the

optimization test. Based on these results, an incubation time of 7 hour in LB medium without NaCl with no dilution of the seawater sample was used for rapid detection in subsequent analysis of seawater samples, as it resulted in the greatest rate of increase in viral titer over hours of enrichment. However, there were no significant differences among all conditions when compared statistically (One-way ANOVA, $p>0.05$). These conditions were compared to standard overnight incubation in subsequent analyses of seawater samples.

a. b.



14. Figure 5.1 Results of conditions of test of media, dilution factor, and culture (incubation) time for somatic coliphage analysis in seawater. Errors bar show standard deviations of triplicate sample assays. The concentrations in Tryptic soy broth (TSB) are shown in a, and the concentrations in Luria broth (LB) without NaCl are shown in b.

Comparison of somatic coliphage detection by rapid enrichment culture and overnight enrichment culture Although the rate of increase in viral titer was measured to optimize enrichment conditions, the endpoint of the enrichment assay is the presence or absence of a zone of lysis when the enrichment is spotted onto a lawn of host bacteria. Both 7 hour and overnight (18~ 24 hours) incubation times were compared by the spot plate method for their

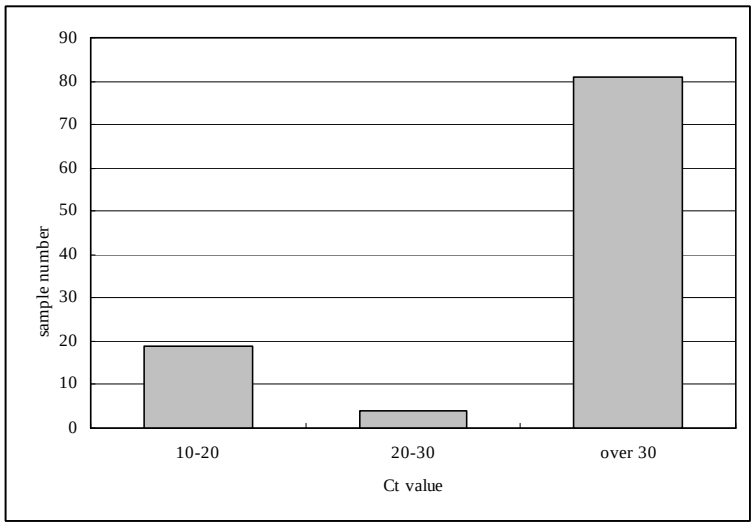
ability to detect positive enrichments. A total of 115 seawater sample were compared with rapid and overnight enrichment culture. Table 5.1 shows the proportion of samples from each incubation time with positive zones of lysis at the end of incubation. After overnight enrichment, 84 of 115 samples were positive for the presence of somatic coliphage, but only 70 of 115 samples were positive after 7 hour enrichment. However, when compared statistically, there is no significant difference between coliphage positivity of 7 hour enrichment and overnight enrichment (Chi-square test, p=0.0680).

16. Table 5.1 Comparison of positivity of somatic coliphage detection after rapid enrichment culture (after 7hr enrichment), overnight enrichment culture, and real-time PCR in seawater samples

Somatic coliphage enrichment	Rapid culture	Overnight culture	Real-time PCR
Positive no. of sample/Total no. of sample for analysis	70/115	84/115	104/115
Percent positivity (%)	61	73	91

Application of molecular detection methods to seawater After overnight enrichment, seawater samples were analyzed for the presence of the *Microviridae* family of somatic coliphages by real-time PCR. PCR results for *Microviridae* were compared to total somatic coliphage results using the spot plate MPN method. Table 5.1 shows the distribution of positive samples between overnight enrichment culture and real-time PCR methods. The methods of coliphage detection by enrichment and real-time PCR are different. For the enrichment method, the target for detection is infectious members of the somatic coliphage group and the infectivity concentration unit is MPN/100ml. However, the actual enrichment volume subjected to spot plate analysis is 10ul per spot. For real-time PCR method, the target for detection is the *Microviridae* family of somatic coliphages and concentration is in PCR units per 4ul. Therefore, it is possible that the differences in the target coliphages and the sample volumes between methods contribute to the differences in the results of two methods.

There were positive results in real-time PCR methods even though overnight enrichment methods showed negative results. With real-time PCR, 104 of 115 samples were positive for *Microviridae*, where only 84 of 115 samples were positive for total somatic coliphage using the spot plate detection method. When considering the differences in positivity between two methods, there is a statistically significant difference (Chi-square test, p=0.001). This difference in number of positive samples detected could be because of different detection limits between the enrichment MPN (Most Probable Number) method and real-time PCR method. The majority of positive samples showed lower Ct values by real-time PCR methods, which suggest that the samples have relatively low virus concentrations (Figure 5.2).

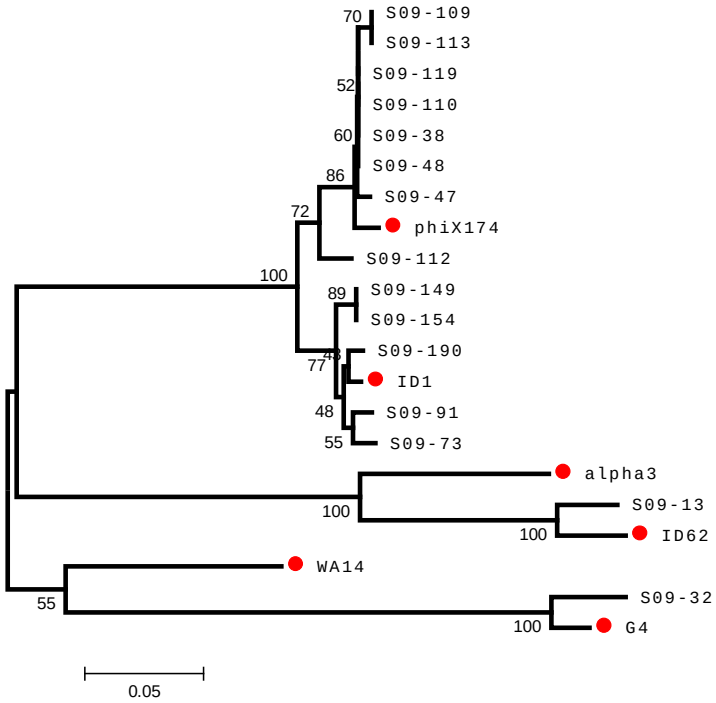


15. Figure 5.2 Distribution of Ct values of real-time PCR method applied to seawater sample after overnight enrichment culture of total 115 samples.

After real-time PCR analysis, 88 seawater samples from overnight enrichment culture were selected for analysis using *Microviridae* family specific conventional PCR. Of 88 samples, 17 were positive for the presence of *Microviridae* by conventional group-specific PCR. The majority of samples were negative by conventional PCR although positive by real-

time PCR. When considered statistically, there were significant differences between the positivity rates of two methods (Chi-square test, $p<0.0001$). This difference could be from the differences between detection limits of two molecular methods. To further characterize these positive samples, 15 samples were sequenced. Figure 5.3 shows the results of sequence analysis, which found that most of the isolates were clustered with the phiX174 group of the *Microviridae* family.

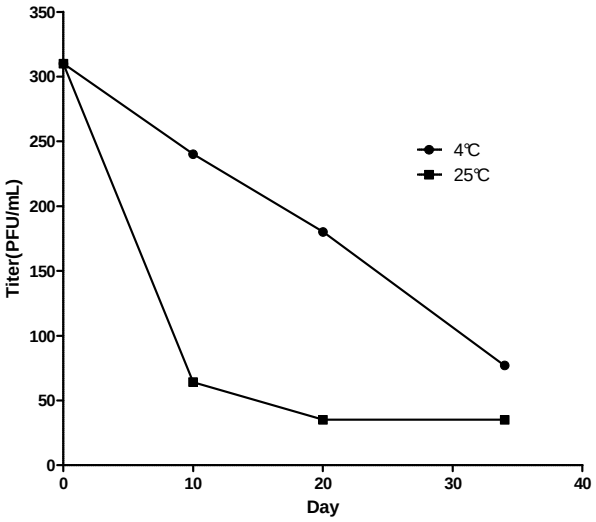
In addition, 15 samples positive by the *Microviridae* family-specific conventional PCR were analyzed by real-time PCR for comparison of Ct values. By real-time PCR, Ct values of 11 of 15 positive samples were between 10 and 30. This suggests that samples with lower Ct values in real-time PCR are more likely to show positive results in conventional PCR. This is consistent with the expectation that lower Ct values are an indication of higher concentrations of viral genome targets for PCR amplification in the sample, which in this case is a specific conserved genome region of the *Microviridae* family.



16. Figure 5.3 Clustering of sequencing results of *Microviridae* family positive isolates from seawater samples using ClustalW2 and MEGA (Version4) program. Dot shows the positive controls which were included in the alignment and clustering. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches

Survival test somatic coliphages in K-water sample Water samples (primary sewage) from Korea (K-water) were held at two temperatures (4°C and 25°C) and sample aliquots were taken at different time points and analyzed for somatic coliphages in order to measure survival. Figure 5.4 shows the changes of somatic coliphage concentrations in the samples over time at the two incubation temperatures. The concentration of somatic coliphages at the beginning of survival test was 310 PFU/ml. At 4°C, the somatic coliphage concentration declined gradually over the experiment period following first order kinetics. However, somatic coliphage concentrations at 25°C were unchanged initially and then declined abruptly after ten days. Also, during the subsequent incubation period of the survival test at

25°C, the concentration of somatic coliphage remained around 50 PFU/ml with little further decline. Hence, the somatic coliphage reduction at 25°C followed bi-phasic kinetics. From this somatic coliphage survival time course study of primary sewage effluent from South Korea, a total of 176 somatic coliphage isolates were collected and archived for subsequent analysis.



17. Figure 5.4 Titer (PFU/ml) changes of somatic coliphage in sewage effluent of wastewater treatment plant from South Korea (K-water) during incubation at two different temperatures of 4 °C (circle) and 25°C (square).

Application of group-specific conventional PCR and real-time PCR methods for K-water sample somatic coliphage isolates to detect the *Microviridae* family The conventional PCR and real-time PCR methods were applied to detect the *Microviridae* family among the time course isolates from South Korea wastewater. The optimized conventional PCR and real-time PCR methods described in the previous chapter were applied to the detection of the *Microviridae* family among somatic coliphage isolates. Table 5.2 shows the results of conventional PCR and real-time PCR analysis of isolates from sewage

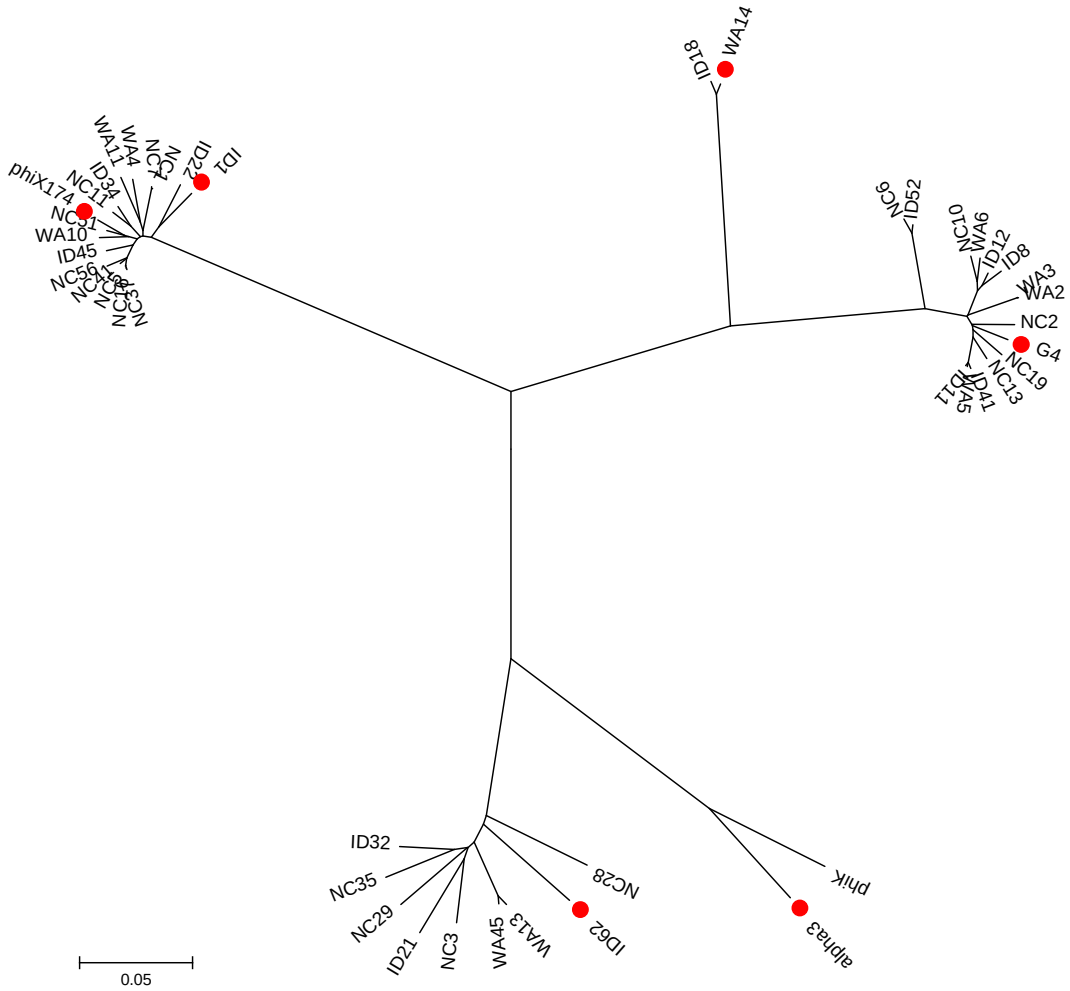
effluent of the South Korea wastewater treatment facility. By the conventional PCR method, the *Microviridae* positive samples increased gradually over time especially at 25°C. Also, by real-time PCR, the majority of sample isolates were *Microviridae* positive by Day 20 at both 4 and 25°C, and by Day 34 they were 100% *Microviridae*-positive at both 4°C and 25°C.

17. Table 5.2 Presence of the *Microviridae* family among somatic coliphage isolates from K-water wastewater treatment plant effulent as a function of sample incubation time based on *Microviridae* analysis using group-specific conventional PCR and real-time PCR.

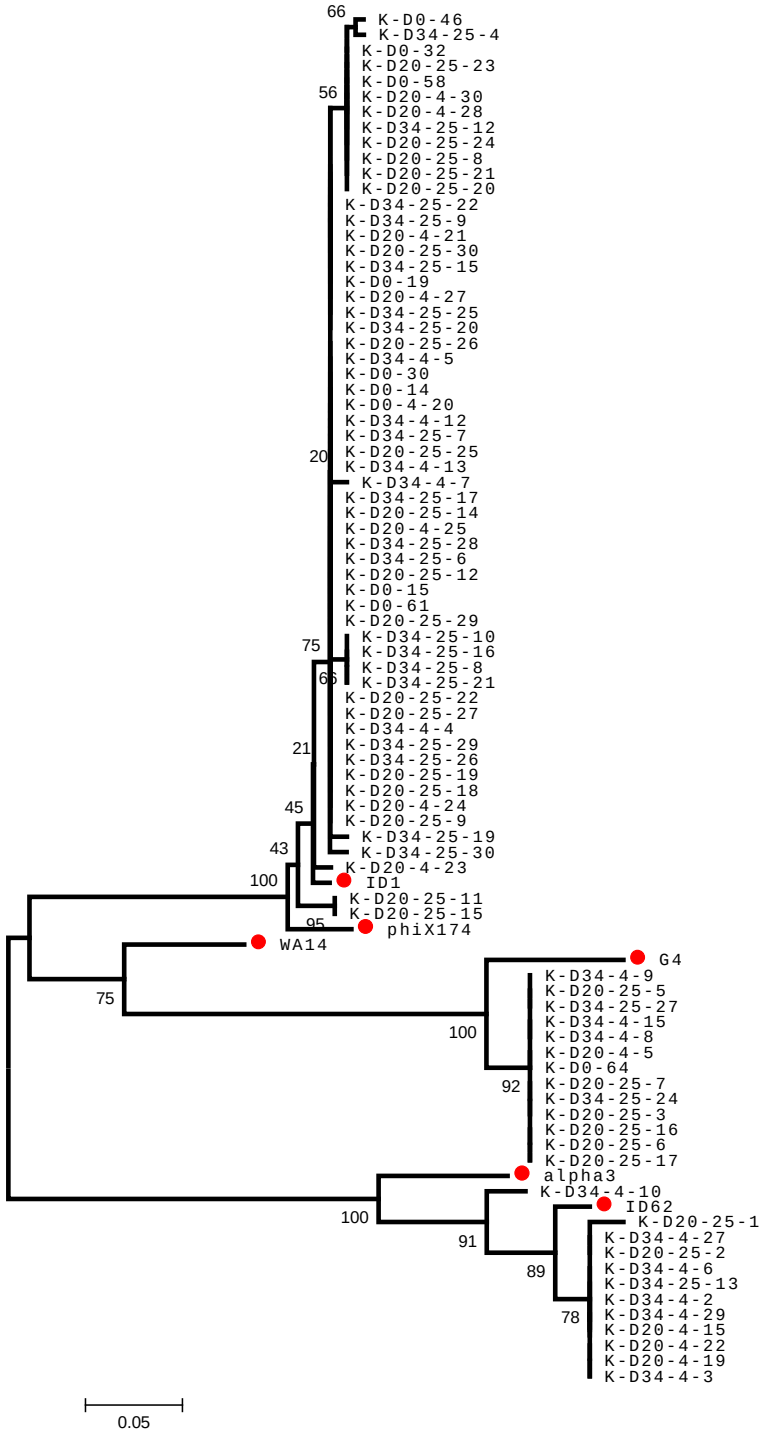
Methods	Incubation day (Temperature for survival test)	0	20 (4°C)	20 (25°C)	34 (4°C)	34 (25°C)
Group-specific Conventional PCR	No. *Microviridae* Positives/ Total No. Isolates	9/56	15/30	29/30	16/30	25/30
	Percent (%) Positive	16	50	97	53	83
Real-time PCR	No. *Microviridae* Positives/ Total No. Isolates	24/56	24/30	30/30	30/30	30/30
	Percent (%) Positive	43	80	100	100	100

Sequencing and phylogenic analysis of isolates for the *Microviridae* family positive sample of K-water To further characterize somatic coliphage isolates from primary sewage effluent of South Korea, all positive isolates by group-specific conventional PCR for the *Microviridae* family were subjected to sequence analysis. The reference strains of the *Microviridae* family of each subgroup shown in figure 5.5 were included for sequence analysis of somatic coliphage isolates (Figure 5.5). There were five subgroups in cluster analysis of the strains in the *Microviridae* family, namely PhiX174, G4, alpha3, WA14, and ID62. Subgroup PhiX174 was predominant among somatic coliphage isolates from South Korea wastewater. However, compared to OWASA somatic coliphage isolates, South Korea sample isolates were more diversified in their subgroups (Figure 5.6). Most of OWASA

isolates mapped to the PhiX174 subgroup (Figure 4.3) while there were some K-water isolates that mapped to G4, alpha3, ID62, and PhiX174 subgroup.



18. Figure 5.5 Cluster analysis of all *Microvridae* strains in the NCBI website using the MEGA4 program. By branch division, there are 5 subgroups in the *Microviridae* family. Red dots show the reference strains of each subgroup in the *Microviridae* family for subsequent sequencing analysis of K-water somatic coliphage isolates: reference strains PhiX174, ID62, alpha3, G4, and WA14. ID1 are included as these strains were the best blast matches for sequence analysis of the wastewater isolates.



19. Figure 5.6 Clustering of sequencing results of *Microviridae* family-positive coliphage isolates from K-water sample using ClustalW2 and MEGA (Version4) program. Dot shows the positive controls which were included in the alignment and clustering. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

Application of real-time PCR method for *Microviridae* coliphage analysis to groundwater samples from CA A total of 16 groundwater samples from California were tested for somatic coliphages by the enrichment culture method (rapid and overnight culture), followed by spot plating with 10ul per spot and real-time PCR. For real-time PCR, a 100ul volume of enriched sample was extracted and 4ul of the extract was used for PCR amplification. Table 5.3 shows the results of somatic coliphage detection in the 16 groundwater samples. All groundwater samples were positive for somatic coliphages by real-time PCR even though the majority of enrichments were negative for somatic coliphages by spot plating. This could be from differences in the sample volume, detection target, and detection limit between enrichment and real-time PCR methods. Also, the differences of positivity between rapid enrichment culture (7 hours) and overnight culture (~18-24 hours) method were not statistically significant (Chi-square test, p=0.6942).

18. Table 5.3 Comparison of positivity for somatic coliphage detection after rapid enrichment culture (after 7hr enrichment), overnight enrichment culture, and real-time PCR in groundwater samples

Methods	Rapid culture	Overnight culture	Real-time PCR
No. positive no. samples/No. total sample analyzed	4/16	5/16	16/16
Percent Positivity (%)	25	31	100

Discussion

The somatic coliphage isolates from sewage effluent from OWASA were analyzed using the optimized CLAT assay in an effort to identify their specific somatic coliphage family and strain. Among 160 isolates, 20 unidentified isolates by molecular methods were tested by optimized CLAT assays to detect those that were T4-like (*Myoviridae*), N4-like (*Podoviridae*), and in the *Siphoviridae* family. However, none of the isolates yielded positive

CLAT assay results when tested using agglutination particles coated with antisera against T4, N4, and T1 coliphages. However, there were positive isolates for the T4 subgroup of *Myoviridae* family by conventional group-specific PCR analysis in previous studies of these isolates (see Chapter 3). This difference in detection could be due to differences in the lower detection limit of each method. The T4 subgroup detection limit by conventional PCR was approximately 3×10^3 PFU/10ul (Table 3.5). However, the detection limit by CLAT for T4 was 3×10^6 PFU/10ul (Table 4.8), which is a far less sensitive detection limit.

Previous studies of somatic coliphage families in environmental waters have been conducted primarily by electron microscopy. Pedroso *et al.* (1995) found somatic coliphages belonging to three families, *Myoviridae, Siphoviridae, and Podoviridae*, in different surface and ground water samples in Brazil. Investigation of sewage to identify somatic coliphage families by Ackermann *et al*. (1983) showed that *Myoviridae* and *Podoviridae* were the predominant somatic coliphage families present. Muniesa *et al.* (1999) studied the relationship between the morphology of somatic coliphages and their persistence in environment waters. They found that *Myoviridae*, followed by *Siphoviridae*, were the most abundant families in raw sewage, treated sewage, and an upper river water site. However, members of the *Siphoviridae* family became more abundant in a downstream river site.

The distribution and ecology of somatic coliphage has not been studied adequately to characterize and quantify somatic coliphage populations on the basis of their taxonomy. Therefore, investigation of regional variations of somatic coliphage family presence in sewage would provide better understanding of the distribution and prevalence of families of somatic coliphage populations. Such investigations are now possible by using the molecular methods developed in this study. Primary sewage effluent from a wastewater treatment plant

in South Korea (K-water) was investigated for the survival of somatic coliphages over time using new methods for conventional PCR and real-time PCR detection of the *Microviridae* family. The same methodology was also applied to OWASA sewage effluent from Chapel Hill, NC. These analyses made it possible to examine the similarities, differences and regional variations of the *Microviridae* family in somatic coliphage populations of sewage effluents from the two geographically disparate wastewater treatment plants. It was found that the overall concentrations of somatic coliphages decreased at both test temperatures: 25°C and 4°C. However, after 30 days, the somatic coliphage concentrations in these wastewaters remained at about 50 PFU/ml at both temperatures for >90% somatic coliphage survival. At 25°C, survival kinetics were bi-phasic, with a lower survival rate (more rapid inactivation) initially and greater survival rates (less rapid inactivation) later in the experimental period. This result suggests that some members of the somatic coliphage population of sewage survive longer than others over time. After completion of these survival tests, the archived somatic coliphage isolates were tested to detect the presence and genotypes of *Microviridae* family members in the somatic coliphage populations of these sewage samples.

The developed real-time PCR and conventional PCR methods for detection of the *Microviridae* family were applied to K-water samples. The extent of positive results for the *Microviridae* family was very similar to that for the OWASA somatic coliphage isolates. The fraction of total coliphage isolates belonging to the *Microviridae* family increased over the time course of the survival test when detected by both real-time PCR and conventional PCR methods. Of 176 total isolates, 53.4% were positive for the *Microviridae* family by conventional PCR and 78.4% were positive for the *Microviridae* family by real-time PCR

method. By the real-time PCR, all (100%) of the somatic coliphage isolates on day 20 at 25°C and on day 34 at both 25°C and 4°C, were positive for the *Microviridae* family. These results suggest that members of the *Microviridae* family are more persistent among K-water somatic coliphage isolates as was also found for OWASA isolates.

In addition to detection and identification by PCR analysis methods, nucleotide sequencing was conducted on the isolates positive for the *Microviridae* family by conventional PCR. There were 80 *Microviridae* family-positive isolates from K-water by conventional PCR (out of a total of 176 somatic coliphage isolates) that were subjected to sequencing analysis, Results for the best blast match of the 80 sequenced K-water sewage isolates indicated that they were genetically more diverse than the isolates from OWASA sewage. *Microviridae* strains ID1, NC6, ID62, NC11, ID34, NC35, NC28, and WA11 showed matching results with the K-water isolates. However, >50% of isolates were best matched with ID1 which was the same result for the OWASA isolates.

Even though all somatic coliphage families were not included in the identification and typing of somatic coliphages in sewage by molecular or immunoassay methods, the *Microviridae* family showed high prevalence and persistence in regionally different sewage samples (Chapel Hill, NC USA and Chungbuk, South Korea). In contrast to previous studies in which the *Siphoviridae* and *Myoviridae* family were abundant in environmental waters (Ackermann *et al*., 1983; Pedroso *et al*., 1995; Muniesa *et al.*, 1999), in this present study the *Microviridae* were found to be the taxonomically identifiable predominant family in sewage that persisted and became even more predominant over the time course of survival tests in this study. The reasons for these differences in prevalent and persistent somatic coliphage virus families could be from the different methods to detect and identify somatic coliphage

strains and families in water. Previous studies relied mainly on electron microscopy for detection and identification, while the present study relied mainly on molecular analysis of family- and subgroup -specific viral genome targets for detection and identification. Electron misroscopic detection and identification of small, non-enveloped icosahedral viruses like those of the *Microviridae* family is difficult due to the small size of these viruses and the resemblance to other particles of similar size and shape in sample matrices.

To further test the application of the developed real-time PCR methods to seawater, Malibu Beach (CA, USA) seawater samples were examined for somatic coliphages. An optimized enrichment liquid culture method was initially applied to seawater samples to detect somatic coliphages, and the real-time PCR method was then applied to the enriched sample. Many samples were positive for the *Microviridae* family. Also, a newly developed method for conventional group-specific PCR amplification of the *Microviridae* family was applied to the same enriched seawater samples. PCR-positive sample by conventional PCR were sequenced to determine the genetic characteristics of the isolates from seawater and examine their diversity. Coliphages mapping to subgroup NC51 of the *Microviridae* family were the most prevalent based on the best blast match among the isolates of 15 seawater samples. This result differs from the previous sequencing results of somatic coliphage isolates from primary sewage effluent of both OWASA and K-water. However, the number of coliphage isolates sequenced was not sufficient to statistically compare differences in *Microviridae* genotypes among them. Nevertheless, the developed PCR methods were successfully applied to somatic coliphage isolates enriched from the seawater and groundwater samples.

In conclusion, a real-time PCR method for the detection and identification of somatic coliphages of the *Microviridae* family was successfully developed and evaluated. This method has the potential to provide rapid results for detection, quantification, and source tracking of feces-specific, abundant and persistent somatic coliphages by detecting this prevalent and persistent family among somatic coliphages in sewage and fecally contaminated water. Also, the development of new and rapid nucleic-based detection and characterization methods facilitates future studies of somatic coliphage ecology and evaluation of the prevalence and persistence of individual somatic coliphage families such as the *Microviridae* and their subgroups as viral indicators for water quality assessment. These molecular methods will also make rapid water quality assessment possible for management decisions if individual somatic coliphages families are verified as reliable indicators of sewage and enteric viral contamination in environmental waters. This study shows that PCR-based methods can be used to identify and classify somatic coliphages in water and municipal sewage, and can be used to expand the body of evidence on the ecology of somatic coliphages and their utility as sewage and viral indicators in studies of relevance to water quality and health.

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CHAPTER 6 Summary, Conclusions, and Recommendations

Summary

This research evaluated somatic coliphages as candidate indicators of sewage contamination and the possible presence of sewage-borne pathogenic human viruses in environmental waters. To investigate the possibility of individual somatic coliphage families (*Microviridae, Myoviridae, Siphoviridae,* and *Podoviridae*) serving as reliable viral indicators in environmental waters, survival tests were performed using prototype strains to determine if the families differ in their relative persistence in water. Experiments in which test waters (reagent grade water and surface water) were spiked with prototype positive strains and incubated at 4 and 25°C showed that *Myoviridae* as represented by T4, *Microviridae* as represented by PhiX174, and *Siphoviridae* as represented by Lambda survived the longest at both temperatures. Based on these results, it is concluded that these virus families are likely to be preferred candidate indicators. Consistent with previous findings for other viruses, virus survival was greater at the lower temperature tested.

For family-level identification of somatic coliphages from primary sewage effluent, family-specific conventional PCR was developed and applied to somatic coliphage isolates. By applying group-specific conventional PCR for each of the 4 major taxonomic families of somatic coliphages, it was possible to document that the taxonomic composition of the somatic coliphage population in primary effluent of sewage changed over time. The

Microviridae family became the most prevalent taxonomic group in the sewage population after several weeks of incubation at 4 and 25°C. Although the majority of identifiable somatic coliphages isolates from primary sewage effluent belonged to the *Microviridae* family, some of isolates belonging to the *Myoviridae* family also were detected; no isolates were identified from the *Siphoviridae* or the *Podoviridae* families. Based on sequencing and NCBI database blast searching and mapping, it was possible to show that all PCR-positive environmental isolates from primary sewage effluent mapped to T4 of the *Myoviridae* family and ID1, Enterobacteria phage PhiX174 subgroup of the *Microviridae* family.

Rapid real-time PCR for detecting the *Microviridae* family as a possible viral indicator was developed and successfully applied to samples of primary sewage effluent, seawater, and groundwater. The primer sets and specific probe of conserved regions of the *Microviridae* family were used for initial testing and optimization of a TaqMan real-time PCR method by application to PhiX174 as a representative of the *Microviridae* family. Using a highly conserved region of the family for primer and probe design contributes to the family specificity of the newly developed real-time PCR methods. The standard curve generated by TaqMan real-time PCR using PhiX174 showed good linearity with over 99% of R-square by correlation analysis and sufficient sensitivity with the lower detection limit of 0.3 PFU/mL, using PhiX174 as a test organism. The generated standard curve was used to detect and quantify members of the *Microviridae* family among somatic coliphage isolates from primary sewage effluent and somatic coliphage enrichment from seawater and groundwater. The real-time PCR method for the *Microviridae* family could be applied either directly to environmental waters or after enrichment with an appropriate host.

Polyclonal antisera were raised against specific type strains representing the individual somatic coliphage families of *Microviridae, Myoviridae, Siphoviridae* and *Podoviridae.* The antisera were raised in rabbits by a commercial laboratory using phage antigens prepared at UNC in the form of partially purifed coliphage stocks inactivated by either UV or pasteurization. The partially purified phage stocks used were chosen as representative strains for each somatic coliphage family: T4 in the *Myoviridae*, PhiX174 in the *Microviridae*, N4 in the *Podoviridae*, and Lambda and T1 in the *Siphoviridae* family. After initial titration of antisera of each family by plaque reduction neutralization, dot blot immunoassay, enzyme-linked immunosorbent assay (ELISA), and western blots were applied to antisera of each family to test their sensitivity and specificity. Anti-PhiX174 serum reacted with antigens associated with the *E. coli* host bacteria, but did not react with the PhiX174 strain itself. Anti-T4 serum also reacted with antigens of the host *E. coli* bacteria, and also reacted with somatic coliphage strains T4 and N4. Anti-T1 reacted with both their *E. coli* host antigens and the representative members of its *Siphoviridae* family (T1, Lambda, and HK97). Anti-Lambda serum reacted with all *E. coli* host antigens and all phage antigens tested. Therefore, there is no target phage family specificity by anti-Lambda antiserum. Anti-N4 serum did not react with their host *E. coli* antigen but only with N4 strain itself.

Anti-T4 and Anti-T1 sera were applied to CLAT assay even though they showed reactivity with their host, *E. coli* B. Anti-T4 serum and Anti-T1serum did not react with *E. coli* CN-13, which was the host used for enrichment of somatic coliphage isolates from water. Therefore, Anti-N4 serum, Anti-T4 serum, and Anti-T1 serum were applied to the CLAT assay directly for N4 (*Podoviridae*), T4 (*Myoviridae*), and *Siphoviridae* family detection.

Both conventional group-specific PCR and real-time PCR for the *Microviridae* family of somatic coliphage were successfully applied to somatic coliphage isolates from time-course samples of a survival study in sewage effluent produced by urban populations in South Korea (K-water) and North Carolina (OWASA). The survival kinetics of somatic coliphages at 4°C and 25°C were similar in both K-water and OWASA sewage effluents. As in OWASA sewage, the *Microviridae* family in K-water became the most abundant members of the somatic coliphage population over time. All isolates from K-Water identified as belonging to the *Microviridae* using conventional PCR were sequenced (80 of 176 total isolates). The best blast matches for the sequencing results were more diverse than the results of OWASA isolates. The best blast matches for the K-water positive isolates in the *Microviridae* family mapped to ID1, NC6, ID62, NC11, ID34, NC35, NC28, and WA11.

The enrichment conditions for somatic coliphages were optimized to decrease the inhibition of coliphage replication in seawater by varying culture media type, enrichment time, and seawater sample dilution with reagent water. After optimization testing, 7 hour incubation of undiluted seawater supplemented with LB medium lacking NaCl was used for rapid detection in subsequent somatic coliphage analysis in seawater samples. After 7 hour and overnight (18~ 24 hours) enrichment of seawater samples, enrichment aliquots were taken to test for somatic coliphage presence by the spot plate method. A total 115 seawater samples were compared with rapid enrichment and overnight enrichment culture. Of samples positive after overnight enrichment, 84% were positive after 7 hour enrichment. After overnight enrichment, samples were taken to detect the *Microviridae* family in the seawater samples by real-time PCR. There were positive results in real-time PCR methods even though overnight enrichment methods showed negative results. This could be because of the

difference of detection limit between enrichment MPN (Most Probable Number) and real-time PCR methods. A total of 16 groundwater samples were tested for somatic coliphages by enrichment culture method (Rapid and overnight culture) and the real-time PCR method. All groundwater samples were positive by the PCR method, though the Ct values of all samples were higher than 35. Also, the differences of positivity between rapid enrichment culture (7 hours) and overnight culture (18 ~24 hours) methods were not statistically significant.

These new approaches provide new tools for the specific and rapid estimation of the occurrence and concentrations of somatic coliphages as fecal indicators of sewage contamination and the possible presence of enteric viruses in water. These detection methods make possible rapid water quality assessments based on detecting one or more of these somatic coliphage families in water. The new methods developed in this study have the potential to be applied for timely regulation and management of water quality, if additional studies document the abilities of somatic coliphages to reliably predict human enteric virus presence in and enteric illness risk from water. Therefore, the new methods of somatic coliphage detection provide a rapid basis for estimating sewage contamination and possibly enteric viral contamination. However, further investigation is needed to determine if somatic coliphage presence and concentrations are predictive of the presence and concentrations of human enteric viruses that cause severe gastrointestinal disease. Such studies would thereby make it possible to determine if somatic coliphages provide adequate biological water quality information in timely manner to estimate viral disease risks and protect public health.

Conclusions

- The results of studies on prototype somatic coliphage survival in seeded waters suggest that based on their greater persistence in water over time, T4 in the *Myoviridae*, PhiX174 in the *Microviridae*, and Lambda in the *Siphoviridae* are possible candidates as fecal indicator viruses in water due to their greater survival compared to T1 in the *Siphoviridae* and T7 in the *Podoviridae*.

- Results of studies of two *Siphoviridae* family type strains, T1 and Lambda, suggest that members of this family may vary in their survival and persistence in water.

- Virus type, water type, temperature, and incubation time were significant predictors of somatic coliphage inactivation ($p<0.0001$) by regression analysis. Although virus type was a significant predictor overall, when each individual virus type was considered separately, PhiX174, Lambda, T1, and T7 were significant predictive variables ($p<0.0001$), whereas T1 was not significant ($p=0.3661$).

- When regression analysis modelling with an interaction variable was assessed the interactions between water type and each individual virus were significant ($p<0.05$), whereas the interactions between temperature and each individual virus were not significant ($p>0.05$). Therefore, water type is a more important predictor than temperature for estimating somatic coliphage reductions.

- Using a conventional, group-specific PCR method developed to identify each of the 4 major taxonomic groups (*Microviridae, Myoviridae, Siphoviridae,* and *Podoviridae*) of somatic coliphages, it was possible to detect and classify individual somatic coliphage isolates.

- Based on persistence and prevalence in environmental waters, phages belonging to the *Microviridae* family appear to be a promising candidate somatic coliphage indicator group for the presence of sewage contamination and the possible presence of human enteric viruses in fecally contaminated water.

- The development of PCR-based molecular typing of somatic coliphages provides improved methods for the study of their ecology and distribution and the investigations of the possible merits of individual somatic coliphage families as viral indicators in water.

- The observed results are not fully representative of the somatic coliphage population in the primary sewage effluent because the conventional group-specific PCR method is not able to detect all the members in four major families of somatic coliphages. This could be from either the characteristics of the sewage or limitations of group-specific conventional PCR for each family.

- A real-time PCR method for the *Microviridae* family was developed using a highly conserved region as the target for designing primers and specific probe for family-specific amplification. Good linearity of the standard curve of TaqMan real-time PCR using PhiX174 as a positive control in the *Microviridae* family demonstrated the reliability of this real-time PCR method.

- The real-time PCR method was more sensitive than a conventional PCR method to detect members of the *Microviridae* family in sewage effluent over the time course of a survival test of somatic coliphages in sewage. The real-time PCR method provided

more rapid and sensitive results for *Microviridae* family detection than group-specific conventional PCR for this family.

- Because there were no significant differences in real-time PCR Ct values between different environmental water matrices compared to RNase free water, the real-time PCR method for the *Microviridae* family was not adversely impacted by nucleic acid amplification inhibititors and therefore can be applied to varied environmental waters without inhibition effects from different water types.

- Attempts were made to raise family-specific antisera that were reactive with all members of a particular somatic coliphage family using representative strains of four families. This was only successful for the *Siphoviridae*. Other antisera reacted with individual strains but not with all family members. This could be due to high family diversity at the protein level, or the strains chosen may not be representative of the entire virus family.

- Based on specificity of antisera at individual strain or family level, CLAT assay was optimized and applied to detect T4 strain in the *Myoviridae*, N4 strain in the *Podoviridae* and the *Siphoviridae* family among the somatic coliphage isolates from OWASA. There were no positive results for T4, N4, or the *Siphoviridae* among somatic coliphages isolated from North Carolina sewage effluent. This could be due to the lower detection limit of the CLAT assay (ranging from 2×10^3 to 3×10^6 PFU/10ul) or the characteristics of the somatic coliphage population in sewage from OWASA.

- Neither the real-time PCR method nor the antibody-based CLAT assay method could detect of all individual somatic coliphage families. However, for the real-time PCR method, the highly conserved region used for family-specific amplification of the family was effective for the *Microviridae* family. The inability to develop CLAT assays was because there was no sufficient family specificity of developed antisera for the *Microviridae, Myoviridae,* and *Podoviridae* families.

- Somatic coliphages of the *Microviridae* family showed higher persistence and were the most abundant members of the somatic coliphage populations in K-water primary sewage affluent as confirmed by group-specific conventional PCR and real-time PCR.

- The results for both OWASA and K-water somatic coliphage isolates suggest that the *Microviridae* family is the most persistent and abundant family of somatic coliphages in sewage of different geographic origins.

- The majority of *Microviridae* isolates in K-water sewage mapped to the following previously characterized strains: ID1, NC6, ID62, ID34, NC35, NC28 and WA11, which were isolated from locations in South Korea. The cluster analysis of the *Microviridae* isolates from K-water showed three subgroups of PhiX174-like, alpha3-like, and G4-like compared to those from OWASA, which showed only one main subgroup of PhiX174-like. Therefore, it could be concluded that the *Microviridae* strains found in K-water are different from the *Microviridae* strains found in OWASA, NC and are more diversified in genetic relatedness.

- After optimizing enrichment conditions to decrease incubation times in seawater, 7 hour enrichment was still required for reliable somatic coliphage detection, which is still too long for real-time or same day decision-making.

- Real-time PCR was found to be a more sensitive method than spot plate culturing for detection of somatic coliphages in enriched seawater and groundwater samples based on a higher positivity rate of real-time PCR than spot plate culturing.

- The basis for somatic coliphage detection is different for real-time PCR and enrichment culture spot-plating. The real-time PCR detects the *Microviridae* family in a conserved genetic region and the spot plate culturing after enrichment detects infectious somatic coliphages, with no distinction among different families. In contrast, the real-time PCR method estimates the presence of the *Microviridae* family, which appears to be the predominant somatic coliphage family in sewage and fecally contaminated water, based on their relative persistence and abundance.

Further study

By family-specific conventional PCR methods developed in this study, the majority of identifiable somatic coliphage isolates from primary sewage effluent belonged to the *Microviridae* and *Myoviridae* families and no isolates were identified from the *Siphoviridae* or the *Podoviridae* families. Further studies are recommended to determine if these results are characteristic of the somatic coliphage make-up of primary sewage effluent or if there could be differences in those families present based on regional and seasonal effects.

Reliable viral indicators should be present when human enteric viruses are present in contaminated water. Therefore, further studies are needed to determine if there is such an association between the *Microviridae* family of somatic coliphage and the human enteric viruses in sewage and sewage-contaminated waters. Such further investigation will provide better understanding of their associations.

Further improvements of rapid CLAT assays for somatic coliphages are needed. The CLAT assay is an easier, faster, and more field applicable method than real-time PCR. It is recommended that efforts be made to reduce enrichment culture time, to eliminate antisera cross-reactivity with *E. coli* bacteria and possibly other non-somatic coliphage antigens by purification, to improve detection specificity of different somatic coliphage families, and to improve (increase the lower) detection limit of target somatic coliphage families. A recommended approach to achieve broad but sensitive family-specific detection is to produce polyclonal antisera by serial immunization of the same animal with purified somatic coliphage antigens representative of each major subgroup of a family. This approach has the potential to provide high-titered family-specific antisera that is potentially broadly reactive enough to detect all members of the family, regardless of sub-group or strain. Such improvements in the CLAT assays for somatic coliphages are recommended to provide a more robust system to investigate individual somatic coliphage families as candidate viral indicator groups for sewage contamination and for the presence and concentration of human enteric viruses.