CHAPTER 8

# Bacteriophages as Fecal Indicator Organisms

Suresh D. Pillai

## 1.0. INTRODUCTION

Bacteriophages, also known as phages, are viruses that infect bacterial cells. They were first described by Frederick Twort in 1915 and then in 1917 by Felix d'Herelle. D'Herelle named them bacteriophages because of their ability to lyse bacteria on the surface of agar plates; the word *phage* is derived from the Greek for "eating" (Flint et al., 2000). A variety of bacteriophages that infect different bacterial cells have been isolated. In fact, bacteriophages exist for all known bacterial species (Joklik, 1988). Based on their structural and genetic diversity, phages have been classified into different families as shown in Table 8.1. The characteristics of some well-known bacteriophages are shown in Table 8.2.

Bacteriophages are widely distributed in the environment and have been found in groundwater (Pillai and Nwachuku, 2000; Borchardt et al., 2003), river water (Hot et al., 2003; Skraber et al., 2004), irrigation waters (Ceballos et al., 2003; Mena and Pillai, 2003), wastewaters (Ackermann and Nguyen, 1983; Ottoson and Stenstrom, 2003; Nelson et al., 2004), oceans (Paul et al., 1997; Jiang et al., 2001; Jiang and Chu, 2004), and bioaerosols (Dowd et al., 1997; Espinosa and Pillai, 2002). They have also been found in shellfish (Humphrey and Martin, 1993; Croci et al., 2000) and on the surfaces of vegetables and herbs such as carrots and parsley (Endley et al., 2003a, 2003b).

This chapter focuses on the applicability of bacteriophages as indicators of fecal pollution or contamination. Most of the available information on the use of bacteriophages as indicator organisms pertains to wastewater and drinking water microbiology and hence most of the examples cited in this chapter are from these disciplines. Only recently have reports started appearing in the food microbiology literature pertaining to bacteriophages as indicators of fecal contamination on foods (Hsu et al., 2002; Endley et al., 2003; Munian-Mujika et al., 2003; Allwood et al., 2004).

## 2.0. INDICATOR ORGANISMS

Ashbolt et al. (2001) have suggested that there could be different classes of indicator organisms depending on their ultimate application as process indicators, as fecal indicators, or as index or model organisms. Process indicators are organisms that are used to demonstrate the efficiency of a particular

**Table 8.1** Classification of Bacteriophages

| *Group or Family* | *Genus* | *Type Member* | *Morphology* | *Envelope* | *Type of Nucleic Acid* |
|---|---|---|---|---|---|
| Corticoviridae | Corticovirus | PM2 | Isometric | No | Supercoiled dsDNA |
| Cystoviridae | Cystovirus | ϕ6 | Isometric | Yes | 3 segments of dsRNA |
| Inoviridae | Inovirus<br>Plectrovirus | fd<br>Acholeplasma phage | Rod | No | Circular ssDNA |
| Leviviridae | Levivirus<br>Allolevirus | MS2<br>Qβ | Icosahedral | No | Linear positive strand RNA |
| Lipothrixviridae | Lipothrixvirus | Thermoproteus phage 1 | Rod | Yes | Linear dsDNA |
| Microviridae | Microvirus<br>Spirovirus | ϕX174<br>Spiroplasma phages, MAC-1 | Icosahedral | No | Circular ssDNA |
| Myoviridae | | T4 | Tailed | No | Circular dsDNA |
| Plasmaviridae | Plasmavirus | Acholeplasma phage | Pleiomorphic | Yes | Circular dsDNA |
| Podoviridae | | Coliphage T7 | Tailed | No | Linear dsDNA |
| Siphoviridae | Lambda phage group | Coliphage lambda | Tailed | No | Linear dsDNA |
| Sulpholobus shibatae virus | | SSV-1 | Lemon-shaped | No | Circular dsDNA |
| Tectiviridae | Tectivirus | PRD1 | Icosahedral | No | Linear dsDNA |

ss, single stranded; ds, double stranded.

**Table 8.2** Characteristics of Selected Bacteriophages[a]

| *Phage* | *Common Host* | *Head Size (nm)* | *Tail Length/ Structure (nm)* | *Structure* | *Nucleic Acid Type* | *Nucleic Acid Structure* | *Mol. Weight ($\times 10^6$)* |
|---|---|---|---|---|---|---|---|
| T1 | *E. coli* | 50 | 150/simple tail | Icosahedral | dsDNA | Linear | 27 |
| T2, T4, T6 | *E. coli* | 85 × 110 | 110/complex tail with fibers | Prolate icosahedral | dsDNA | Linear | 110 |
| T3, T7 | *E. coli* | 60 | 15/short tail | Icosahedral | dsDNA | Linear | 25 |
| T5 | *E. coli* | 65 | 170/short tail | Icosahedral | dsDNA | Linear | 80 |
| λ | *E. coli* | 64 | 140/simple tail | Icosahedral | dsDNA | Linear | 32 |
| P22 | *S. typhimurium* | 61 | 20/complex tail | Icosahedral | dsDNA | Linear | 29 |
| SPO1 | *B. subtilis* | 90 | 200/complex tail | Icosahedral | dsDNA | Linear | 85 |
| PM2 | *Pseudomonas* | 60 | None | Icosahedral, lipid-envelope | dsDNA | Circular | 6 |
| ϕX174 | *E. coli* | 27 | None | Icosahedral | ssDNA | Circular | 1.7 |
| M13 | *E. coli* | 7 × 900 | None | Filamentous | ssDNA | Circular | 2.1 |
| MS2, Qβ | *E. coli* | 24 | None | Icosahedral | ssRNA | Linear | 1.2 |
| ϕ6 | *Pseudomonas phaseolica* | 65 | None | Icosahedral, lipid containing envelope | dsRNA | 3 linear segments | 9.5 |

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.
[a] Adapted from Joklik, 1988.

**Table 8.3** Ideal Characteristics of a Pathogen Indicator Organism[a]

1. Should be present when pathogens are present and absent when pathogens are absent.
2. The persistence and growth characteristics of the indicator should be similar to the pathogens.
3. The indicator organism should not multiply in the environment.
4. The ratio between the indicator organism and the pathogen should be constant.
5. The indicator organism should be present in greater concentrations than the pathogens in contaminated samples.
6. The indicator organism should be resistant or more resistant to adverse environmental factors, disinfection, and other treatment processes as pathogens.
7. The indicator organism should be non-pathogenic and easy to quantify.
8. The tests for the indicator organism should be easy and applicable to all types of samples.

[a] Modified from Goyal, 1983.

man-made or natural process; fecal indicators are organisms that are used to infer the possible presence of fecal contaminants in a milieu; and index or model organisms are indicators for the presence of a particular pathogen. Goyal (1983) recommended that indicator organisms chosen as fecal contamination indicators or pathogen indicators should satisfy certain specific criteria (Table 8.3). More recently, the IAWPRC Study Group on Health Related Water Microbiology (1991) suggested that an ideal indicator organism should meet five specific criteria as listed in Table 8.4.

Over the past years, different organisms have been proposed as indicators of fecal contamination including fecal coliforms, *Escherichia coli*, enterococci, bacteriophages, and so forth (Berg et al., 1978; Kibbey et al., 1978; Fiksdal et al., 1985; Jin et al., 2004). However, studies have repeatedly shown that bacterial indicators are not true representatives of all possible fecal contaminants, especially of the enteric viruses (Berg et al., 1978; Gerba, 1987; Wait and Sobsey, 2001; Duran et al., 2003) and that bacteriophages may be a better indicator of such pollution (Havelaar et al., 1986; Gerba, 1987;

**Table 8.4** Ideal Characteristics of a Fecal Contamination Indicator Organism

1. The indicator organism should occur consistently and abundantly in fecal material, preferably exclusively in human wastes.
2. The indicator organism should not multiply in the environment or foods.
3. The indicator organism should have an ecology in the environment and foods similar to that of pathogens.
4. The indicator organism should respond to environmental stresses similar have resistance to environmental stress similar to the pathogens.
5. The laboratory analysis for the detection of the indicator organism should be simple and relatively inexpensive.

Havelaar, 1993; Hsu et al., 2002; Cole et al., 2003; Endley et al., 2003a; Hot et al., 2003).

The use of coliphages as indicators of fecal pollution is based on the assumption that their presence in water samples denotes the presence of bacteria capable of supporting their replication. The advantages of coliphages as indicators of enteric viruses (therefore fecal pollution) include their relative similarities in size, transport, survival or persistence patterns, and densities in sewage or septic samples. In addition, coliphages are found in relatively high numbers in the environment, do not readily multiply in the environment, and can be assayed at a fraction of the cost of a typical pathogenic enteric virus assay. Their relatively long persistence in the environment and resistance to common disinfectants make them conservative indicators of fecal contamination. The use of phages that infect *Bacteroides fragilis* has also been considered as good fecal pollution indicator. *Bacteroides fragilis* is an obligate anaerobe found in high concentrations in human feces, and the presence of phages that infect these bacteria is considered to be indicative of fecal pollution (ISO, 1999; Puig et al., 1999; Gantzer et al., 2002; Duran et al., 2003; Lucena et al., 2003).

## 3.0. COLIPHAGES

The use of coliphages as indicators of not only fecal contamination but also of enteric bacteria and viruses was suggested almost 20 years ago by Gerba (1987). Coliphages can be broadly categorized into somatic and male-specific (or $F^+$) coliphages. The former are phages that infect *E. coli* by attaching directly to the cell wall, whereas the latter infect the *E. coli* host by attaching to a specific bacterial appendage termed sex-pilus or F-pilus. After attachment, the viral nucleic acid is injected into the host bacteria through these appendages. Muniesa et al. (2003) reported that only a negligible number of naturally occurring bacteria can serve as potential hosts for somatic coliphages.

Coliphage numbers in humans, cows, and pigs range from approximately $10^1$ to $10^7$ pfu per gram of feces (Dhillon et al., 1976; Osawa et al., 1981; Havelaar et al., 1986) and they are almost always present in raw sewage at $10^4$ to $10^6$ pfu/ml. Coliphages do not generally multiply in the environment because of the need for a live host for multiplication. However, there is a theoretical possibility that somatic coliphages can multiply in certain environments where *E. coli* can also multiply (e.g., in raw sewage). This is considered to be a drawback for the use of coliphages as indicators (Muniesa et al., 2003). However, to date there have been no reports of coliphage multiplication in natural environments probably because the temperatures required for efficient phage infection and replication (i.e., >30°C) are rare in the open environment. In addition, optimum phage and bacterial densities and bacterial physiological conditions needed for phage replication are rarely found in the natural water environment (Muniesa and Joffre, 2004).

### 3.1. Somatic Coliphages

Somatic coliphages have been found in sewage-contaminated tropical waters but not in pristine waters (Toranzos et al., 1988). They have also been detected in storm-water runoff (Davies et al., 2003), graywater samples (Ottoson and Stenstrom, 2003), and in bioaerosols around wastewater treatment plants and animal-rearing operations (Espinosa and Pillai, 2002). Suan et al. (1988) found somatic coliphages to be highly correlated with fecal coliforms in tropical waters in Asia but, in Chile, Castillo et al. (1988) found low correlation between somatic coliphages and fecal coliforms and a weak correlation between somatic coliphages and total coliforms. In fact, the coliphage-to-coliform ratio has been found to vary widely in sewage, secondary effluent, and river water and is influenced by environmental temperature (Bell, 1976). In one study, Carducci et al. (1999) did not find statistical relationship between the presence of coliphages and other organisms in bioaerosols collected around a wastewater treatment facility. However, the bioaerosol sampling employed in that study was not designed for effective coliphage capture. Skraber et al. (2004) reported that somatic coliphages were less sensitive to environmental stresses than the thermotolerant coliforms and thus were much more reliable indicators of fecal contamination. Torella et al. (2003) have reported that somatic coliphages of *Salmonella* spp. and *E. coli* were more resistant than fecal coliforms to freezing and cold temperatures (4°C).

The degradation of viral genomes of somatic coliphages has been found to be much more similar to that of infectious viruses, suggesting that somatic coliphages can be used as reliable indicators of fecal or pathogen contamination even when molecular methods are used to detect them (Skraber et al., 2004). Of the 68 surface-water samples positive for somatic coliphages ($10^3$–$10^4$ pfu/L) in France, only 2 were positive for enteroviruses by virus isolation in cell cultures and 60 were positive for enteroviruses by molecular methods (Hot et al., 2003).

In a survey of shellfish in Spain, Muniain-Mujika et al. (2003) reported somatic coliphages in 90% of the shellfish samples (n = 60) collected over a 6-month period. Of the 36 shellfish (mussels) samples harvested from 3 sampling sites in the Adriatic Sea, Croci et al. (2000) detected somatic coliphages in 78% of the samples with concentrations ranging from 0.4 MPN/g to 110 MPN/g. Significantly, none of the samples were positive for male-specific coliphages, only 4 samples were positive for enteroviruses, and 13 samples were positive for hepatitis A virus. Lucena et al. (1994) have reported that somatic coliphages and Bacteroides phages appear to have the lowest decay rates compared with others in shellfish growing areas around Spain. Hsu et al. (2002) detected somatic coliphages in 88% (n = 8) of market samples of poultry as compared with male-specific coliphages that were detected in 63% of the samples. Ceballos et al. (2003) detected somatic coliphages ($10^3$ to $10^5$ pfu/100 ml) in a river that was being used as an irrigation water source in Brazil.

### 3.2. Male-Specific Coliphages

Male-specific ($F^+$) coliphages are coliphages that infect *E. coli* via the bacterial sex-pilus, the genes for which are located on the F-plasmid. The $F^+$ coliphages can be RNA-containing (FRNA phages) or DNA-containing coliphages (FDNA phages). The specificity of male-specific coliphages to infect only the *E. coli* cells that produce the F-pilus is critical because the F-pilus is produced only at temperatures near 37°C or higher. Thus, the potential for male-specific coliphages to replicate in the environment, where temperatures are rarely around 37°C, is negligible. However, the potential for these coliphages to multiply in environments where the ambient temperatures may reach 37°C during certain periods of the year needs to be explored.

The FRNA phages are a relatively homogenous group of small, icosahedral coliphages belonging to group E (Leviviridae). Because FRNA phages are morphologically similar to many of the enteroviruses, have similar persistence and transport characteristics in water, and are equally resistant or more resistant to chlorination than enteroviruses, they have attracted considerable attention as an indicator of enteric viral contamination (Shah and McCamish, 1972; Duran et al., 2003; Shin and Sobsey, 2003). Although humans do not excrete large numbers of FRNA phages in their feces, they are found in significantly large numbers in human sewage (Osawa et al., 1981; Furuse et al., 1983; Havelaar et al., 1986), leading some to believe that FRNA phages may multiply in raw sewage. The use of male-specific coliphages as fecal indicators circumvents the technical complexities and costs involved in screening for enteric viral pathogens yet provides some assurance about the absence of viral fecal contaminants (Havelaar, 1993a, 1993b; Hsu et al., 1995; Allwood et al., 2003). Allwood et al. (2003) have recently suggested that the presence of male-specific coliphages may be a strong indicator for the presence of noroviruses in water samples. They based their conclusions on the survival patterns of *E. coli*, male-specific coliphages and feline calicivirus in dechlorinated water stored for 28 days at 4°C, 25°C, and 37°C.

Male-specific coliphages have also been isolated from foods and food production/processing facilities. Espinosa and Pillai (2002) reported on the detection of male-specific coliphages from bioaerosols within poultry (broiler) houses; both FRNA and FDNA phages were detected in samples collected within the buildings and on window ledges just outside the buildings. Hsu et al. (2002) detected FRNA phages in 5 of 8 (63%) "market-ready" samples of poultry meat products. Further, they were able to monitor the presence of male-specific coliphages and other indicator organisms through the evisceration, washing, and chilling processes and showed that FRNA phages were reduced by more than 1 $\log_{10}$ pfu. Endley et al. (2003) found male-specific coliphages on cilantro and parsley in the absence of *E. coli*, indicating that it may be useful to monitor male-specific coliphages in addition to *E. coli* when screening for fecal contamination of herbs. Of the 18

retail samples each of cilantro and parsley, 9 cilantro samples (50%) and 7 parsley samples (39%) were positive for male-specific coliphages.

In another study, Endley et al. (2003a) demonstrated the value of male-specific coliphages as an additional fecal contamination indicator when screening vegetables such as carrots. In this study, FDNA phages were detected in 25% of the carrot samples as compared with *E. coli* and *Salmonella*, which were present in only 8% and 4% of the samples, respectively. One of the salient features of this study was the observation that the occurrence of the fecal indicator organisms was random and that the contaminated sample may at times be positive for only one or two of the indicator organisms (Table 8.5).

Croci et al. (2000) observed that neither *E. coli* nor male-specific coliphages were reliable indicators for the presence of enteric viruses in mussels from the Adriatic sea. They report that out of 36 mussel samples that were analyzed, only 3 samples (8%) were positive for male-specific coliphages. Though these 3 samples were also positive for HAV, only 1 of these 3 samples was positive for enteroviruses. Muniain-Mujika et al. (2003) studied the comparative presence of pathogenic viruses and indicator organisms in shellfish. Out of 60 shellfish samples that were collected in 3 "human impacted" areas, 47% were positive for human adenoviruses, 19% were positive for enteroviruses, and 24% for HAV. The FRNA phages were present in 17 out of 60 (28%) shellfish samples. Enteroviruses, HAV, and human adenoviruses were repeatedly detected in samples that were negative for *E. coli*. Though only four of the FRNA positive samples (6.7%) were positive for HAV, enteroviruses, and human adenoviruses, the data strongly suggest that *E. coli* occurrence is not correlated with the occurrence of viral indicators or pathogens.

Humphrey and Martin (1993) have reported that somatic coliphages (rather than male-specific coliphages) have value as a fecal contamination indicator of virus removal during relaying of Pacific oysters. Their conclusions were based on the die-off of male-specific coliphages in oyster tissues.

Dore and Lees (1995) have reported on the persistence of male-specific coliphages in the digestive glands of environmentally contaminated bivalve molluscs even after depuration, indicating that these coliphages can proba-

**Table 8.5** Microbial Indicator Organisms and Pathogens on Carrots Obtained from Different Locations[a]

| | *Number of Positive Samples* | | |
|---|---|---|---|
| *Organism* | *Field* | *Truck* | *Packing Shed* |
| Male-specific coliphages | 1/25 (4%) | 4/25 (16%) | 14/25 (56%) |
| *E. coli* | 0/25 (0%) | 4/25 (16%) | 2/25 (8%) |
| *Salmonella* | 1/25 (4%) | 2/25 (8%) | 0/25 (0%) |

[a] Modified from Endley et al., 2003a.

bly serve as a conservative indicator to verify that all traces of fecal contamination have been removed by depuration. Allwood et al. (2004) reported a stronger correlation between the survival of feline calicivirus and the FRNA phage MS2 than between *E. coli* and any other viral pathogen on the surfaces of leafy salad vegetables. These results also support the notion that FRNA phages can serve as a conservative indicator when evaluating pathogen intervention strategies.

### 3.3. Bacteroides Phages

*Bacteroides fragilis* is an obligate anaerobic bacterium found in high concentrations in human feces, and hence the presence of phages that infect these bacteria is considered to be indicative of human fecal contamination (Chung and Sobsey, 1993; Grabow et al., 1995; Jofre et al., 1995; ISO, 1999; Lucena et al., 2003). It should be realized, however, that the numbers of Bacteroides phages will vary depending on the host strain used and on the geographical region from where samples originated (Cornax et al., 1990; Araujo et al., 1997; Puig et al., 1999). Using *B. fragilis* strain RYC20, Muniain-Mujika et al. (2003) reported high correlation between Bacteroides phages and human enteric viruses in shellfish. Lucena et al. (1994) report that the Bacteroides phages have one of the lowest decay rates among indicator organisms in shellfish and that the fate of Bacteroides phages released into the marine environment mimics that of human viruses more than any other indicator organisms. Bacteroides phages are more resistant to conventional drinking water treatment processes than even male-specific coliphages and clostridia (Jofre et al., 1995) and they are also resistant to thermal treatment processes that are commonly used in sewage and sludge treatment (Mocé-Llivina et al., 2003), indicating that these phages may be the most conservative indicators.

## 4.0. DETECTION OF BACTERIOPHAGES

Due to the increased interest in using coliphages and Bacteroides phages as contamination indicators, the methods to detect them have been constantly improving. One of the key issues that confront bacteriophage detection in food and water is the appropriate sample volume for analysis. Because enteric viruses are normally recovered using very large sample volumes, the current trend in bacteriophage analysis, at least in the research laboratories, is to employ large sample volumes as well. For the recovery, detection, and enumeration of phages, a variety of different methods have been reported based on sample volume, sample processing method, and the host bacteria used (Pillai and Nwachuku, 2002).

### 4.1. Membrane Filtration Method

Sobsey et al. (1990) have reported on a bacteriophage detection method based on membrane filtration. In the original method, the host bacterium

*Salmonella typhimurium* WG49 was employed to detect F-specific coliphages. However, because the $F^+$ plasmid has been found to be unstable within the host bacterium, the authors acknowledged that there could be interferences (false positive) from somatic coliphages. This led to the identification of *E. coli* ATCC 15597 as the host strain for detecting male-specific coliphages. The protocol is based on adding $MgCl_2$ to the water sample followed by filtration of a defined volume (usually 1,000 ml) of the sample through a 0.45-µm pore-size filter. The phages attached to the filter are eluted in a high pH buffer after which the eluate is neutralized and plated on the appropriate host strain. The use of 0.03% tetrazolium dye aids in the detection and enumeration of the phage-induced plaques due to the contrast the dye provides.

### 4.2. U.S. EPA Information Collection Rule (ICR) Method

The U.S. EPA published a standardized procedure for the enumeration of somatic and male-specific coliphages for use in the Information Collection Rule (USEPA, 1996). In this procedure, large volumes of water sample (usually >1,000 L) are passed through a positively charged 1MDS filter after which the adsorbed phage particles are desorbed using a high-pH buffer. There are obviously constraints related to the volume of the sample that can be passed through the filter depending on the amount of suspended solids in the sample. Lingering concerns about the stability of phages to high-pH conditions employed in this protocol have forced researchers to explore alternate sample processing strategies.

### 4.3. ISO Methods

The ISO (International Organization for Standardization) method for enumeration of F-specific RNA phages is the official standard for ISO, which stipulates that the samples have to be collected, transported, and stored according to specific guidelines. This method also recommends the use of *S. typhimurium* WG49, *E. coli* K-12 Hfr, or *E. coli* HS (pFamp) as the host and includes a preconcentration step for samples that may harbor low numbers of bacteriophages. This protocol is designed for all types of water samples and can be adapted for use with food samples provided careful thought is given to sample processing and purification. The salient feature of the protocol is that the method suggests confirmatory steps when sampling new sources, when there is an unexplained overabundance of F-specific phages, or when there is an indication that somatic phages are being isolated (Pillai and Nwachuku, 2002). The basic protocol consists of using semi-soft TYG (tryptone yeast extract glucose) agar amended with a calcium-glucose solution to which 1 ml of the undiluted or diluted sample is added, mixed, and poured over a bottom-agar plate. The confirmatory tests involve the use of RNase (40 µg/ml) amended TYGA media. The ISO method can also be adjusted for use with samples containing high bacterial background using nalidixic acid–resistant *E. coli* strain CN-13 (ATCC 70078), also known as WG5. For the detection of somatic coliphages in samples with low back-

ground bacterial counts, the use of *E. coli* strain C (ATCC 13706) as the host strain has been recommended. Another highlight of the ISO protocol is the built-in confirmatory steps in phage detection when sampling new sources, when there is an unexplained overabundance of F-specific coliphages, or when there is an indication that somatic phages are being isolated. The confirmation steps include the use of RNAse for selectivity. The ISO method for the enumeration of bacteriophages infecting *B. fragilis* uses *B. fragilis* RYC 2056 (ATCC 70078) as a host. The primary advantage of this host is that, although the bacterium is an obligate anaerobe, *it does not require* anaerobic handling conditions. Only the incubation has to be carried out under anaerobic conditions.

### 4.4. U.S. EPA Methods 1601 and 1602

The two U.S. EPA methods 1601 and 1602 are performance-based methods designed and optimized for qualitative and quantitative detection of somatic and male-specific bacteriophages. These protocols have been extensively tested in round-robin laboratory and field tests and are being considered to be included in the pending EPA Ground Water Rule. Method 1601 is a two-step enrichment procedure for the qualitative detection of male-specific and somatic coliphages (USEPA, 2000a) and has been used to detect coliphages in carrots, cilantro, parsley, and bioaerosols (Endley et al., 2003a, 2003b; Espinosa and Pillai, 2002). The method can be used with either 100-ml or 1,000-ml sample volumes. The use of large sample volumes, as mentioned earlier, increases the probability of detecting low levels of fecally associated phages. Somatic coliphages are detected using *E. coli* CN-13 (a nalidixic acid–resistant mutant of *E. coli* ATCC 700609) as the host strain, while male-specific coliphages are detected using *E. coli* F-amp (an ampicillin-streptomycin–resistant mutant of *E. coli*). The principle underlying this method is the addition of host bacterium, $MgCl_2$, and concentrated broth medium into the sample followed by overnight incubation at 37°C. After this incubation, aliquots of the "enriched" culture are spotted on plates containing pre-prepared lawns of host bacteria. The plates are then incubated overnight and the resulting plaques are counted. It should be realized, however, that plaque counts cannot be used for quantitative purposes because they have originated from enriched samples.

The U.S. EPA Method 1602 is a quantitative detection protocol (USEPA, 2000b). This method also uses *E. coli* CN-13 and *E. coli* F-amp as hosts for detecting somatic and male-specific coliphages, respectively. However, this method is capable of handling only 100-ml sample volumes. The method involves the addition of high titer host bacterium, double-strength agar medium and $MgCl_2$ to the 100-ml sample, after which the entire contents are poured into 5 to 10 Petri dishes. After overnight incubation, the plaques are counted and tallied across different plates and the results reported as pfu/100 ml.

Recent improvements to these protocols include the use of confirmatory steps for plaque visualization by "picking" plaques from the original isola-

tion plates and respotting them on fresh spot plates (Sobsey et al., 2004). The enrichment method has been found to be extremely valuable for detecting low levels of phages in large sample volumes (Sobsey et al., 2004).

### 4.5. Colorimetric Method

Colorimetric, presence/absence methods for coliphage detection have also been reported (Ijzerman et al., 1993). The method is based on the induction of β-galactosidase by *E. coli*. As a result of coliphage infection/replication, the bacterial cells are lysed, and β-galactosidase hydrolyses the yellow chromogenic substrate that develops into a distinct red color in coliphage-positive samples. Coliphage-negative samples will remain yellow.

## 5.0. BACTERIOPHAGES FOR TRACKING SOURCES OF CONTAMINATION

In addition to detecting the presence of fecal contamination, it is also extremely important to identify the sources of fecal contamination. Only if sources are identified would it be possible to develop remediation approaches to limit the exposure of the environment to fecal contaminants. A number of studies over the recent past have attempted to come up with tools to detect sources of fecal contamination. Indicators such as *E. coli*, enterococci, and bacteriophages and molecular methods such as pulsed-field gel electrophoresis (PFGE), ribotyping, and BOX-PCR have been proposed for source-tracking purposes (Lu et al., 2004; Meays et al., 2004). The PFGE protocol involves a specialized electrophoretic separation of the total genome after restriction digestion with specific enzyme(s). The BOX-PCR protocol involves the selective amplification of BOX sequences within enterobacteria using specialized PCR primers. Ribotyping involves the hybridization of 16S and 23S rDNA sequences as a method of differentiating bacterial subtypes.

Male-specific RNA coliphages have some unique characteristics that permit them to be used for tracking sources of fecal contamination. Phylogenetically, F-specific RNA coliphages fall into four subgroups (Furuse, 1987). Male-specific RNA coliphages are composed of serogroups I through IV. Subgroups I and II are related and form the major group A while subgroups III and IV are very similar and form the major group B. Strains isolated from human feces usually are in group II and III, whereas groups I and IV are usually found in animal feces (Osawa et al., 1981; Furuse, 1987). Cole et al. (2003) have recently reported on the distribution of different subgroups and genotypes of RNA and DNA coliphages. Municipal wastewater samples had high proportions of $F^+$ DNA coliphages and group II and III $F^+$ RNA coliphages. Bovine wastewater samples, on the other hand, though containing a large proportion of $F^+$ DNA coliphages, harbored a majority of group I and IV F+ RNA coliphages. Swine wastewaters harbored equal proportions of $F^+$ DNA and RNA coliphages. Group I and III $F^+$ RNA coliphages were the most common types of RNA coliphages in swine wastewaters. The $F^+$

RNA coliphages (groups I and IV) were present in large numbers in waterfowl feces. Though there was a statistically relevant association between genotypes II and III with human excreta and genotypes I and IV with animal/bird excreta, Schaper et al. (2002) have questioned whether they can be used for absolute distinction between human and animal sources. This was based on the detection of genotype II phages in poultry, cattle, and pig feces and genotype III phages that were reported for the first time in their study. Nevertheless, the understanding of the distribution of genotypes and serotypes in waste streams has paved the way for using male-specific RNA coliphages as indicators for detecting the source of fecal contamination, although antisera for male-specific RNA coliphages are not readily available and some isolates are difficult to serotype. Genotyping of male-specific coliphages with oligonucleotide probes has been found to be a feasible alternative to serotyping (Hsu et al., 1995; Brion et al., 2002). In addition to male-specific coliphages, the use of Bacteroides phages has also been suggested to detect fecal pollution from human sources. Grabow et al. (1995) reported that out of *Bacteroides fragilis* phages were detected in 13% (n = 90) of human fecal samples but were absent in fecal samples from a variety of animals. Thus, the detection of Bacteroides phages is indicative of fecal pollution from human sources.

## 6.0. SUMMARY

The distribution and occurrence of bacteriophages in source water such as rivers and aquifers have been extensively studied over the past years. There are a number of published articles describing the survival characteristics of bacteriophages in natural and man-made or engineered environments. However, our understanding of the occurrence of bacteriophages in foods is rather limited. Other than a few recent publications, there is a serious lack of understanding of the occurrence, distribution, and survival kinetics of these organisms in foods of animal and plant origin. We are currently unsure of the behavior of these coliphages and Bacteroides phages within foods during food processing and the various pathogen intervention strategies that foods are often subjected to. We are unsure for what types of foods these indicator viruses are robust indicators of fecally associated viral contaminants. We need to determine the food categories and the food processing systems in which coliphages can be used as fecal contamination or process indicators. In addition, to understand the ecology of coliphages on foods we need methods that can effectively recover coliphages from foods. These methods should be easy and efficient to use so that phage levels can be used for the estimation of microbial risk.

The technologies for coliphage detection are relatively mature, but processing protocols to recover coliphages from foods are scant. Although a few published protocols exist for recovering coliphages from certain herbs such as cilantro and parsley, concerted efforts are needed to develop methods for

recovering coliphages from various types of vegetables, fruits, salads, and meat and meat products. Rapid methods to characterize the isolated coliphages in terms of their genotype need to be developed so that information about the potential sources of pathogens can also be obtained in parallel. Currently, the methods available for genotyping are restricted to research laboratories. Recent technological advances in micro-array, micro-fluidics, and biosensor technologies need to be exploited to develop user-friendly methods for the specific and sensitive detection and characterization of indicator viruses.

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