

Chapter 35

Indicators of Waterborne Viruses

Robert H. Armon

Abstract Enteric viruses excreted by humans and animals may reach water resources and cause large outbreaks. Drinking water is one of the essential global life elements for humanity. However, some of our resources are contaminated with viruses and indicators for continuous monitoring have been developed. The classical ones are coliforms and fecal coliforms that are still the iron standard for water indicator monitoring (see Chap. 34). In the last decades, bacteriophages have been suggested as potential indicators of enteric viruses and many studies showed their potential as such mainly due to their comparable resistance to water processes such as disinfection. In this chapter, the indicator role of bacteriophages in water is critically reviewed and discussed.

Keywords Enteric viruses • Water pollution • Human origin sewage • Fecal-oral transmission • Enterobacteriaceae • *E. coli* • Coliforms • Bacteriophages • F-male specific phages • Somatic phages

35.1 Background

Human enteric viruses, which by definition are transmitted via the fecal-oral route, are the main waterborne group of viruses that pose a real public health hazard (Estes et al. 2006). Thus far, enteric viruses have been divided into eight families (Table 35.1). The most relevant ones are hepatitis A and E, enteroviruses, rotaviruses, caliciviruses, astroviruses, and enteric adenoviruses, which may cause the respective severe diseases: hepatitis, paralysis, meningitis, myocarditis and heart anomalies, fever, gastroenteritis, conjunctivitis, and respiratory disease (Fig. 35.1).

Enteric viruses are excreted in the feces of infected patients (10 % of the population can shed $\sim 1 \times 10^6$ particles/g of feces, at any given time) and due to contamination of different water sources, i.e., rivers, lakes, effluents, land runoff, estuaries, and groundwater, may infect people via faulty septic systems, sewage outfall, urban and agricultural runoff, wastewater discharge from vessels, and in

R.H. Armon

Faculty of Civil and Environmental Engineering, Technion (Israel Institute of Technology),
Haifa 32000, Israel

e-mail: cvrrobi@tx.technion.ac.il

Table 35.1 Properties and clinical characteristics of major human waterborne and food enteric viruses

Family (genus)	Common name	Size (genome)	Envelope	Incubation/illness duration (days)	Contaminated source	Season	Symptoms
Picornaviridae (enterovirus)	Polio, Coxsackie A & B, echo, human enterovirus (types 68 to 71)	28 nm (ssRNA)	No	4–35/7–14	Water and shellfish	Mostly winter	Cardiomyopathy, meningitis, CNS motor paralysis, gastroenteritis
Picornaviridae (kobuvirus)	Aichivirus	28 nm (ssRNA)	No	39 h/2–4	Shellfish	All seasons	Gastroenteritis
Picornaviridae (hepatovirus)	Hepatitis A virus	28 nm (ssRNA)	No	15–50/7–14 (to several months in serious cases)	Food-borne	Late spring to early summer (in Hong-Kong)	Hepatitis
Hepeviridae (hepevirus) ^a	Hepatitis E virus (formerly non-A non-B hepatitis)	34 nm (ssRNA)	No	21–56/7–28	Mainly water	Winter	Hepatitis
Rotaviridae	Rotavirus	70 nm (dsRNA)	No	2–6/5–7	Mainly water		Gastroenteritis (diarrhea)
Adenoviridae (mastadenovirus)	Adenovirus group F, types 40 and 41	100 nm (dsDNA)	No	8–10/5–12	Water and fecal-oral route	No certain seasonality	Mild diarrhea
Caliciviridae (saprovirus)	Saprovirus	34 nm (ssRNA)	No	1–4/6	Shellfish	Mostly winter	Gastroenteritis
Caliciviridae (norovirus)	Norovirus (Norwalk-like viruses)	34 nm (ssRNA)	No	1–3/4	Water and food-borne	Mostly winter	Explosive projectile vomiting
Astroviridae (mamastovirus)	Human astrovirus	28 nm (ssRNA)	No	1.5–2/1–4	Water and shellfish	Mostly winter	Mild gastroenteritis
Parvoviridae	Wollan, ditchling, Paramatta and cockle agents	25 nm (ssDNA)	No	4–14/7	Food-borne (shellfish)	Late summer to late spring	Gastroenteritis
Coronaviridae	Coronavirus and Torovirus	spherical, 120–160 nm diameter (ssRNA)	Yes	2–5/7	Fecal-oral route or by aerosols of respiratory secretions	Winter and early spring	Severe acute respiratory syndrome (SARS) in humans

^aEmerson (2005)

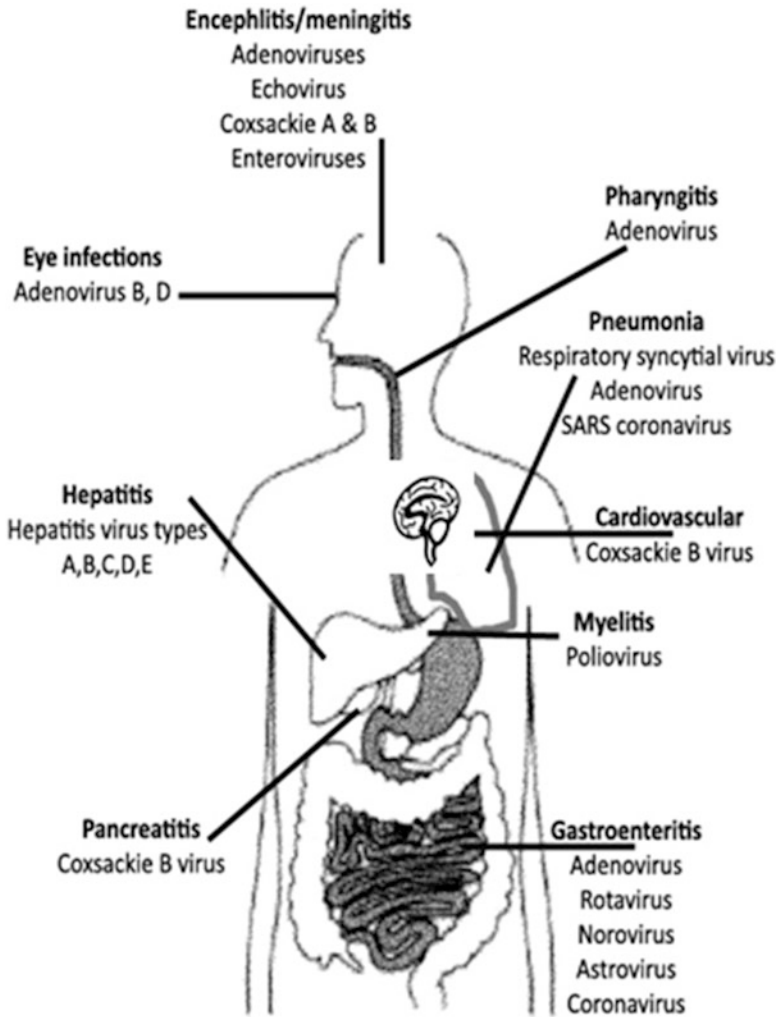


Fig. 35.1 Schematic viral infections in humans and their target organ

most cases, through the use of untreated wastewater for irrigation, typically in less developed countries (Okoh et al. 2010).

Enteric viruses may be transferred in the environment by attachment to particulates present in groundwater, estuarine and seawater, rivers, shellfish grown in contaminated waters, and aerosols emitted by sewage activated sludge processes. Direct human exposure to enteric viruses occurs through various routes, such as irrigation of crops with sewage (intended for water and fertilizers), seafood (shellfish grown in sewage polluted areas), contaminated recreational areas (by means of water sports), and finally, via contaminated potable water. Categorically, studies have shown that waterborne viral disease outbreaks occurred when

the following elements were involved: (a) consumption of untreated surface water; (b) consumption of untreated groundwater; (c) insufficient or sporadic water treatment; (d) faulty public distribution network; and (e) miscellaneous (sewage irrigation, contaminated food, aerosols, etc.). Faulty water treatment and distribution systems contributed to > 80 % of global viral outbreaks.

35.2 Viral Indicators

The best way to monitor viral contamination is through direct detection of the pathogens themselves without using indicators as a proxy. However, this task is strenuous as enteric virus detection and growth methods, where infectivity/viability potential may require cultivation and direct manipulation of pathogenic organisms, are still cumbersome, requiring the processing of large volumes of sample-pathogens where frequently they are present in low concentrations, expensive (tissue culture and molecular methods), and finally time consuming (days). On the one hand, the current molecular methods available in different variations are accurate, relatively fast, and continuously evolving; on the other hand, they are still expensive, require specialized equipment, and do not discriminate between live and dead viral particles (at least, thus far).

Consequently, the indicator system is still the method of choice in virus detection in water sources, essentially due to the procedural simplicity. According to Mossel (1982), when categorizing food marker organisms, there are two imminent definitions that ought to be discriminated: “amongst marker organisms two groups should be distinguished. . .the first one provides information on the risk of occurrence of given pathogens or toxin-formers (not the case of viruses, R.A.) . . . suggesting the name of index organisms for this group” and “a second group of marker organisms used for the purpose of assessing the risk of inadequate bacteriological quality of a general nature that should be called indicator organisms.” As indicated by Mossel (1982), a marker organism “may serve both as an indicator and as an index and even in the same food.” Since the present chapter is dedicated to water viral pollution, it will be useful to unify the two definitions, as viruses fulfil both classifications without losing the main denotation. An indicator must meet several prerequisites to fulfil its task, but only a meronym is essential as such (the most essential ones are indicated by bold letters). It should be:

- (a) **Stable in the environment and under various treatments (survives as long as, or longer, than the pathogens).**
- (b) **Not able multiply outside its host.**
- (c) **Ubiquitous (available in fresh and saline waters).**
- (d) **Exclusively fecal (present at densities related to the severity of fecal contamination or, in other words, it should be associated with the source of the pathogen and be absent in unpolluted areas).**
- (e) **Greater in number/frequency than pathogens.**








- (f) Highly prevalent throughout the year.
- (g) Removed by WWT (wastewater treatment plant) close to pathogens.
- (h) **Simple and inexpensive to count accurately and reproducibly, that is, its analysis procedure should be easy.**
- (i) **Of human or animal origin.**
- (j) A “surrogate” for many diverse pathogens.
- (k) **Not be pathogenic to humans.**

Historically, the conventional indicators of water microbial and viral pollution, some of which are still valid at present, are *Escherichia coli* (total, fecal, and thermotolerant coliforms), *Enterococcus* spp. (fecal streptococci), and *Clostridium perfringens* (sulphite reducing clostridia or spores of sulphite reducing clostridia). However, questions have been raised about the capability of the above indicators to measure water biological quality and predict waterborne viral diseases, primarily because there is a lack of correlation between these indicators and viruses in water samples (Wyer et al. 1995; Borchardt et al. 2003, 2004), and secondly because enteric viruses are more resistant to natural stressors and disinfection processes than are conventional bacterial indicators (Scott et al. 2003). Briefly, some indicators are sensitive to disinfectants and environmental stresses (*Aeromonas*, *Escherichia coli*), while others are too sturdy (*C. perfringens* spores), some present at low numbers in sewage, some are excreted by both humans and animals, some are also pathogens (*E. coli*, *P. aeruginosa*), some are obligatory anaerobes (*Bifidobacterium*), and some do multiply in sewage (most of heterotrophic bacteria). Since the late 1980s, bacteriophages have been regarded as reliable indicators of viral pollution of drinking water via contact with feces or sewage (Hoffmann-Berling and Mazé 1964; Armon 1993; Armon and Kott 1993; Havelaar et al. 1993; Havelaar 1993). However, to be precise, several researchers already suggested the idea of using various bacteriophages as indicators of fecal pollution of water in the 1940s–1950s of the last century (Abdoelrachman 1943; Guelin 1950; Cornelson et al. 1956, etc.). The grounds for this idea are that bacteriophages are also viruses, infecting specifically only bacteria, and the only organism group that closely resembles human viruses, and hence, are worthy candidates as indicators based on their morphology, genomics, and their presence in human or animal feces, and because they are highly resistant to environmental stresses and present in adequate amounts to be enumerated directly without further concentration (Armon et al. 1997; Schaper et al. 2002). Prospective new indicators from this group are somatic coliphages (Kott et al. 1974; IAWPRC Study Group 1991; Armon 1993; Armon and Kott 1996), F⁺-male specific bacteriophages (named also F⁺ RNA coliphages) (Havelaar and Hogeboom 1983; Durán et al. 2002, 2003), and phages specifically infecting *Bacteroides fragilis* bacteria (IAWPRC Study Group 1991; Jofre et al. 1995). Consequently, according to the discrepancies previously described and major indicator prerequisites, bacteriophages have been shown to fit best as viral pollution indicators.

35.2.1 Somatic Coliphages (Potential Indicators of Enteric Viruses)

Frequently, somatic coliphages have been selected from a heterogeneous group of different families' morphology such as *Myoviridae* (i.e., phage T4), *Siphoviridae* (i.e., λ phage), *Podoviridae* (i.e., phage T7), or *Microviridae* (i.e., phage Φ X174) (Table 35.2). Somatic coliphages contain a double-stranded or a single-stranded DNA (ds/ss DNA), encapsulated in a proteinaceous isometric or elongated capsid. One of their major features is the tail (contractile or not), except for *Microviridae* (lacking a tail), that help in infection by attachment to a certain receptor (typically part of a protein, a lipopolysaccharide, the peptidoglycan, teichoic acid, or an exopolysaccharide) on their *E. coli* host's outer membrane, *E. coli* C being the most commonly used as the host (Armon et al. 1988). Other additional hosts have been also reported, such as *E. coli* B, C, C-3000, F-amp, and K-12 derivatives, such as WG21 and W3110, plus several undesigned strains of *E. coli*. However, most of these hosts have restriction enzymes capable of inactivating the invading phages, except *E. coli* C, which does not own a DNA-modifying or restricting system, explaining the high efficiency of plating with somatic phages. Attachment and infection via a receptor located on the *E. coli* cell's outer surface imparted their collective group name "somatic coliphages." Somatic coliphages are regularly found in human sewage and are more prevalent than F⁺ RNA coliphages in marine water and warm waters (Mocé-Llivina et al. 2005; Lovelace et al. 2005; Burbano-Rosero et al. 2011). These bacteriophages show frequent occurrence in human and animal feces (10^2 – 10^8 /g) and wastewater (10^3 – 10^4 /ml), and good environmental persistence, although they are readily inactivated by water treatment processes, with the exception of a few types (Hot et al. 2003; Mocé-Llivina et al. 2005). Kott et al. (1974) found that somatic coliphages were present in wastewater and other fecally-contaminated waters in numbers at least equal to human enteric viruses. Coliphages have been used as water quality indicators for estuarine, sea, fresh, potable, and waste waters, and biosolids (Mocé-Llivina et al. 2003; Sinton et al. 1999), and as indicators of enteric viruses in aerosols from activated sludge, sewage effluents, shellfish, and shellfish-growing water (Fannin et al. 1977; Vaughn and Metcalf 1975), and found to have several limitations. Among these limitations are: their potential multiplication in the environment, as pointed out by many authors (Vaughn and Metcalf 1975; Seeley and Primrose 1980; Parry et al. 1981; Borrego et al. 1990; Grabow 2001); poor correlation of coliphage and enterovirus densities (Nieuwstadt et al. 1991; Wommack et al. 1996); the inability of several coliphages to indicate the presence of solid-associated infective viruses (Moore et al. 1975); the inverse correlation of coliphages and enteric viruses with temperature (Geldenhuys and Pretorius 1989); presence of autochthonous bacteriophages in unpolluted waters (Seeley and Primrose 1980), and finally, host strain variability (Havelaar et al. 1986). Justification for the use of somatic coliphages as sentinels of enteric viruses in wastewater suffers from another potentially important but critical limitation. From the infectivity point of view, this phage group is not

Table 35.2 Bacteriophage families with phage types of particular interest in water quality assessment (as enteric virus indicators)

Group (family)	Characteristics	Representative phages (host)
<i>Myoviridae</i>	dsDNA long contractile tail. isometric or elongated capsids up to 100 nm	T2, T4 (<i>Enterobacteria</i> , e.g., <i>E. coli</i> , <i>Bacillus</i> , and <i>Halobacterium</i>) 
<i>Siphoviridae</i>	dsDNA long non contractile tails ~ 150 × 10 nm isometric capsids up to 60 nm	λ (<i>Enterobacteria</i> , e.g., <i>E. coli</i>), <i>Bacteroides fragilis</i> B40-8, <i>Mycobacterium</i> and <i>Lactococcus</i>) 
<i>Podoviridae</i>	dsDNA short tails isometric capsids up to 65 nm	T7, P22 (<i>Enterobacteria</i> , e.g., <i>E. coli</i> and <i>Bacillus</i>) 
<i>Microviridae</i>	ssDNA without tail isometric capsids ~ 25–30 nm	ΦX174 (<i>Enterobacteria</i> , e.g., <i>E. coli</i> , <i>Bdellovibrio</i> , <i>Chlamydia</i> , and <i>Siroplasma</i>) 
<i>Leviviridae</i>	ssRNA tailless isometric capsids ~25 nm	f2, MS2, GA, Qβ, F1 (F-plasmid bearing bacteria) (<i>Enterobacteria</i> , <i>Caulobacter</i> , <i>Pseudomonas</i> , and <i>Acinetobacter</i>) 
Inoviridae	dsDNA capsids ~800 × 6 nm long and flexible rods	fd, M13 (F-plasmid bearing bacteria) (Bacteria) 
Tectiviridae	dsDNA tailless isometric capsids up to 60 nm lipid membrane below capsid	PRD1, PR722 (Gram-negative bacteria, e.g., <i>Enterobacteria</i>) 

Adapted from Ackermann (2009), Lee (2009)

specific only to *E. coli* species. There is evidence that somatic coliphages may multiply in other species of the family Enterobacteriaceae that comprise the total coliform group (*Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Escherichia* spp.) often found associated with vegetation and biofilms and not restricted to fecal sources. Of these bacteria, the two most common species are *Klebsiella pneumoniae* and *Enterobacter cloacae* (Souza et al. 1972). Therefore, it is possible that somatic coliphages may be produced/present while being unrelated to fecal contamination, and therefore, unrelated to any health risk. Consequently, the use of

somatic coliphages as indicators of fecal pollution and enteric virus presence has serious shortcomings and should be considered as such. Muniesa et al. (2003) studied the factors affecting somatic phage replication using *E. coli* strain WG5. They concluded that there is little chance of somatic coliphages replicating in environmental waters, although it cannot be ruled out completely. The host bacteria and phage threshold densities used by these authors were greater than the highest densities of somatic coliphages and host bacteria reported in most human and animal raw wastewaters. Therefore, they concluded that there are few natural environments in which the densities of non-replicating host bacteria and their physiological status could 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 the animal gut. This potential replication could be affected by several factors, such as the densities of host bacteria and phages, the physiological condition of host bacteria, the dissolved and suspended solids in water, ambient temperature, other bacteria present in water, and additional factors. The replication potential of somatic coliphages in water environments has been considered as a weakness of using somatic coliphages as appropriate viral indicators in water.

35.2.2 F⁺RNA Phages (Synonyms F⁺-Male/F⁺-Specific Phages; F⁺-Specific RNA/DNA Bacteriophages) (Potential Indicators of Enteric Viruses)

F⁺-male specific phages comprise two major groups of bacteriophages (group E and F), according to their genomic composition (RNA or DNA, respectively) and are characterized by attachment and infection of their bacterial host through the pilus (Fig. 35.2) (Singleton and Sainsbury 1993). The pilus is an appendage type, present on the surface of bacterium male strains, encoded by an episomal F-factor. The F-factor is a DNA sequence or plasmid that confers on certain bacteria the ability to produce a sex pilus for conjugation with other bacteria. The F-factor can consequently occur as an independent plasmid. However, it can also integrate into the bacterial genome or chromosome. Hence, bacteria may be classified in relation to the F-factor as HFr (*high frequency of recombination*) when the F-factor is DNA-integrated or F⁺ when separate, both states conferring on a bacterium the so-called “male property.”

F⁺ male phages are a homogenous group (group E) of the family *Leviviridae* with physical properties resembling those of enteroviruses, and group F comprises F⁺ DNA phages of the *Inoviridae* family (Table 35.2). The F⁺ RNA phages (group E) are comprised of non-enveloped, spherical particles with icosahedral symmetry (~26 nm in diameter) containing single-stranded RNA (ssRNA) and divided in four main groups, based on serological and physicochemical properties: group I (phages MS2, f2, BO1 and JP501), group II (phages GA, BZ13, TH1, KU1 and JP34), group

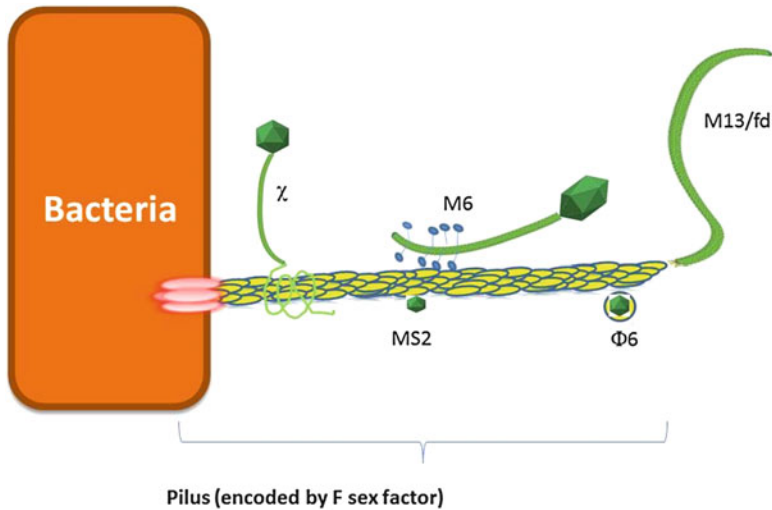


Fig. 35.2 F⁺ pilus as attachment and infection site of various F⁺-male specific phages (see the different attachment positions of F⁺-male specific phages on the same pilus)

III (phages Q β , VK, ST and TW18), and group IV (phages SP, FI, TW19, TW28, MX1, and ID2) (Osawa et al. 1981). The F⁺ DNA phages (Group F) are comprised of a non-enveloped, rod of filaments with a helical capsid with adsorption proteins, on the one hand (7 nm in diameter and 700 to 2,000 nm in length), and a DNA genome, e.g., phage SJ2 (host *Salmonella*), phage fd (host *E. coli*), phage AE2 (host *E. coli*), phage M13 (host *Enterobacter*), phage L51 (host *Acholeplasma*), and phage Pf1 (host *Pseudomonas*), on the other.

The common hosts used to detect F-male specific phages are: *S. typhimurium* phage type 3 Nalr (F = 42 lac::Tn5) (using a male *Salmonella* strain, constructed by the introduction of the plasmid F⁺42 lac::Tn5 into *Salmonella typhimurium* phage type 3) (Havelaar and Hogeboom 1984), *E. coli* K-12 HFr or other F⁺ types, and perhaps the best one, *E. coli* HS[pFamp]R (harboring antibiotic resistance markers, ampicillin on the F_{amp} plasmid, which codes for pilus production, and streptomycin and nalidixic acid on the chromosome) (Debartolomeis and Cabelli 1991). The strain *E. coli* HS[pFamp]R is resistant to coliphages T2 to T7 and Φ X174 and more than 95 % of the phages from environmental samples that plaqued on this strain were F-male specific.

F⁺ RNA phages are intermittently excreted in human and animal feces (up to 10³ PFU/g), but found repeatedly in wastewater (10³–10⁴ PFU/ml). One of the main features of these phages is their environmental multiplication only at temperatures > 30 °C, which is attributable to bacterial pilli formation (male hosts) that occurs only at > 30 °C. These phages also have a relatively high resistance to environmental stresses, such as disinfectants, sunlight, salinity, heat treatment, and water and sewage-treatment processes (Havelaar and Hogeboom 1984; Havelaar and Nieuwstad 1985; Armon et al. 2007). However, according to

their indicator role they have several drawbacks, such as: (1) serotypes may be related to the human/animal origin of fecal pollution; (2) their excretion is not always in sufficiently large numbers to be easily enumerated; and (3) the F⁺RNA coliphages are infectious to bacteria that possess the F-plasmid, and this F plasmid is transferable to a wide range of Gram-negative bacteria, a transferability that raises concern over the lack of *E. coli* specificity (Sobsey et al. 1995).

35.2.3 *Bacteroides fragilis* Phages (Potential Indicators of Enteric Viruses)

Relative new comers in the area of indicator bacteriophages are lytic bacteriophages that are specifically infectious for the anaerobic gut bacterium: *Bacteroides fragilis* (Tartera and Jofre 1987). *Bacteroides fragilis* is one of the most abundant colonic bacterium living and excreted in human feces (up to 10⁸/g). Bacteriophages infecting strains of *Bacteroides fragilis*, *Bacteroides tethaioataomicron*, *Bacteroides ruminicola*, and *Bacteroides ovatus* have been detected in feces and wastewater (Booth et al. 1979; Tartera and Jofre 1987; Klieve et al. 1991; Payán et al. 2005). Bacteriophages infecting *B. fragilis* have been reported to be incapable of replicating outside the gut, merely because of their host strain's requirements, such as anaerobiosis and nutrients, whose absence prevents their replication (Tartera and Jofre 1987). The bacteriophages that infect different *Bacteroides* species have a tail, resembling the morphology of the *Siphoviridae* family: an icosahedric head and a flexible tail (non-contractile, filamentous with fibers) (Table 35.2) (Booth et al. 1979; Klieve et al. 1991; Queralt et al. 2003; Payán 2006). The bacteroides phages' genome consists of double stranded DNA (dsDNA), similar to that of other members of the *Siphoviridae* family (Puig and Gironés 1999; Hawkins et al. 2008). *B. fragilis* phages (grown on *B. fragilis* HSP40 as host) occur only in human feces and do not multiply in the environment, are a relatively homogeneous group, and are relatively highly resistant to environmental stresses, which are qualities of great merit for an indicator; however, they have also some disadvantages, such as: (a) the host strain may not be applicable worldwide (e.g., based on collaborative studies, host *B. fragilis* HSP40 resulted in high phage counts in Southern Europe, Israel, and South Africa, but much lower counts in the USA, UK, and Scandinavia (Armon and Kott 1995; Kator and Rhodes 1992; Puig et al. 1999); host *B. tethaioataomicron* GA17 resulted in high counts in Southern Europe but lower counts in the UK (Payán et al. 2005); *B. ovatus* GB124 resulted in high counts in UK but lower counts in Spain (Payán et al. 2005); *B. fragilis* HB13 resulted in high counts in Spain but lower counts in Colombia (Payán et al. 2005); and the host *Bacteroides* spp. HB73 was good only in Hawaii and *Bacteroides* ssp. ARABA 84 only in Switzerland (Vijayavel et al. 2010; Wicki et al. 2011; Ebdon et al. 2007)); (b) these numbers are relatively low in wastewater (<1–10⁴/ml); and

(c) only 10 % of the population excrete these phages in their feces (albeit large numbers in excretors, $\sim 10^8$ PFU/g) (Tartera and Jofre 1987; Tartera et al. 1989).

In terms of resistance, somatic coliphages, which outnumber phages infecting *B. fragilis* by more than two orders of magnitude, died off faster. Therefore, it can be concluded that *B. fragilis* phages are much more persistent than somatic coliphages and approximately as resistant as F⁺-male specific coliphages (Armon et al. 1997). Lucena et al. (1996) reported high resistance of *B. fragilis* phages as compared to F⁺-male specific coliphages to natural inactivation processes and water treatment (in this case even higher than *Clostridium* spores) (Lucena et al. 1996; Jofre et al. 1995).

Conclusions

It should be emphasized that up to the present time, the only universally accepted indicator of enteric viruses presence in water are still *E. coli* bacteria or, as most laboratories call them, fecal coliforms. As previously presented, *E. coli* is a universal inhabitant of the human gut, excreted in large numbers in feces reaching our sewage. Indeed, the numbers are high enough to be detected easily, but they have a prominent disadvantage: the fecal coliform does not survive well in the environment (e.g., in seawater) and sewage treatment processes (including disinfection), and therefore, as compared with enteric viruses, the *E. coli* indicator is a very fragile one and will decline first. This major problem that tormented environmental virologists for decades has been ameliorated since the 1970s and even earlier, when bacteriophages of different bacteria present in human feces were suggested as better viral indicators (Kott et al. 1974; Armon and Kott 1996). Indeed, bacteriophages seem to fulfil the major prerequisites of viral indicators due to their viral resemblance, fecal excretion, and survival capability characteristics. Table 35.3 summarizes the numbers and/or presence/absence frequency of the three bacteriophage groups isolated from various water sources. From our personal experience and based on others' research, it can be stated that certain bacteriophages can be useful as viral indicators under certain conditions. For instance, the bacteriophage host should be selected from a certain human population, i.e., gut flora can differ between different populations and countries, as previously revealed for the *B. fragilis* host, which is suitable mostly in Europe but not in North America. In the USA, *Bacteroides fragilis* phages were not detected in large numbers in sewage when the Spanish *B. fragilis* HSP40 bacterial host was used for phage detection (Sobsey and Kator, personal communication, 1997). It may be possible that, for bacteriophage detection in a certain geographical area, it will be necessary to isolate primarily a well-defined bacterial host. Furthermore, for each type of water contamination, we should look at one or several

(continued)

Table 35.3 Comparison of the three bacteriophage groups (coliphages, F⁺-male specific coliphages, and *B. fragilis* phages) their presence/isolation frequency/reduction in various water sources

Source	Coliphages	F ⁺ -male specific phages	Bacteroides <i>fragilis</i> phages	Reference
Surface water (river, lakes, ponds, etc.)	34–100 % positive samples	31.8–100 % positive samples	36.4–100 % positive samples	Jofre et al. (1995)
	Enteric viruses 0–55 %	Enteric viruses 0–55 %	Enteric viruses 0–55 % <i>B. fragilis</i> HSP40 (host) Detected in 72 % of water and sediment samples while enteroviruses were detected in only 56 % of those samples	Chung and Sobsey (1993), Tartera et al. (1988)
Ground water	Low concentration	Low concentration	Low concentration	Leclerc et al. (2000)
Water treatment plant reduction ^a (prechlorination-flocculation-sedimentation)	Log 2.6–5.6	Log 2.3–5.2	Log 2.2–2.9	Jofre et al. (1995), Bradley et al. (1999), Kott et al. (1974)
	Enteric viruses: >2.9–>3.4	Enteric viruses: >2.9–>3.4 High resistance	Enteric viruses: >2.9–>3.4	
Brackish Water Salinity (0.1–30 ppt) and Marine water >30 ppt	3 PFU/ml 6.0 MPN/100 ml <1 to 3.4 × 10 ³ PFU/100 ml (in seawater)	0.050–682 MPN/100 ml	> 10 PFU/ml	Madhusudana and Surendran (2000), Love et al. (2010)
Feces	4.3 × 10 ³ PFU/g	<1–6.25 PFU/g, 1–10 ⁵ PFU/g (<1 year old infants)	7 × 10 ¹ –PFU/g	Leclerc et al. (2000)
			24–2.4 × 10 ⁸ /g 0–2.4 × 10 ⁸ /g	Gino et al. (2007)
	Found in humans and animals	Found in humans and animals	Frequency : present in 10–11 % of fecal samples (only humans)	Gantzer et al. (2002) Tartera and Jofre (1987)

(continued)

Table 35.3 (continued)

Source	Coliphages	F ⁺ -male specific phages	Bacteroides fragilis phages	Reference
Urban Sewage or STP	$3.6 \times 10^1 - 1.59 \times 10^4$ /ml	$10^2 - 10^4$ plaque-forming units (pfu) ml ⁻¹ . ~ 10^5 PFU/100 ml	5.3×10^3 /100 ml	Dhillon et al. (1970), Gino et al. (2007)
Effluents	1.4×10^3 PFU/l	10^3 to 10^4 PFU/100 ml	0.8 to 13 PFU/l	Gantzer et al. (1998), Debartolomeis and Cabelli (1991)
Oxidation ponds	$2-3 \times 10^3$ PFU/ml Poor correlation with enteroviruses	300– 10^4 PFU/ml	?	Gino et al. (2007)
Sediments	$>10^6 - >10^7$ PFU/100 ml	$>10^5 - >10^7$ PFU/ml	$>10^4 - >10^5$ PFU/100 ml <i>B. fragilis</i> HSP40 (host). Detected in 72 % of sediment samples, while enteroviruses were detected in only 56 %	Chung and Sobsey (1993), Tartera et al. (1988), Araujo et al. (1997)
Shellfish	Weak correlation	Significantly related to Norwalk-like viruses, less to HAV, adenovirus, enterovirus	<i>B. fragilis</i> RYC2056 (host) Less frequently detected	Formiga-Cruz et al. (2003) Chung et al. (1998)

^aDecimal reduction, decrease in logarithms. Numbers indicate the decimal reduction calculating the value of phages present in finished water using Thomas' equation for the calculation of the most probable number (MPN) for long series of data (De Man 1975). MPN is the number that makes the observed organisms concentration (λ) most probable, expressed by Thomas' equation:

$$\sum_{j=1}^k \frac{g_j m_j}{1 - \exp(-\lambda m_j)} = \sum_{j=1}^k t_j m_j$$

where \exp is e^x , K is the dilutions number, g_j is the test positive numbers in the j th dilution, m_j is the amount (volume or weight) of the original sample in each test volume in the j th dilution, and t_j is the number of tubes in the j th dilution (if tubes used). The equation is generally solved by iteration.

(continued)

groups of bacteriophage types in order to define a certain correlation with sewage or fecal pollution.

Bacteriophages have been intensively studied for almost 60 years as indicators of viral pollution. Nevertheless, governmental regulations rarely specify their use as such and again there is not yet a consensus in the scientific community on their merit. Reviewing the literature, we gradually came to the conclusion that there is no one indicator or index microorganism that can fulfil perfectly the definition previously described. It is our opinion that, similar to the case of the coliform/fecal coliform indicator, in the case of which it was decided to include certain bacterial families as well as to apply a highly defined group, such as fecal coliforms (usually referred to as thermotolerant *E. coli*), as a more specific indicator, bacteriophages will require similar fine-tuning in order to fulfill their real role as indicators.

The use of bacteriophages as indicators started with the broad-spectrum group of bacteriophages infecting *E. coli* host strains (erroneously termed coliphages, as some of them infect other Enterobacteriaceae as well), which resulted in confused conclusions about their indicative potential. Furthermore, there has been no one universally accepted bacterial host, and each laboratory uses its own selected strain. It is clear that this inconsistency prevented an objective comparison of the experimental results among the various laboratories and led to doubts about bacteriophages as potential indicators of water pollution by sewage or fecal material (Gerba 1987).

For the last 30 years, scientists have examined various specific bacteriophages groups, i.e., *Serratia marscens* phages, cyanophages, F⁺-male specific RNA phages, and *B. fragilis* phages (Stanley and Cannon 1977; Smedberg and Cannon 1976), that might fit the indicator role for specific types of pollution. Havelaar et al. (1993) emphasized this idea by showing that F⁺-male specific RNA phages are an adequate model for enteric viruses in fresh water. However, even this publication excluded raw and biologically treated sewage, due to lack of correlation between the presence of these phages and enteric viruses. Kamiko and Ohgaki (1993) substantiated the results of Havelaar et al. (1993) by showing that Q β , an F⁺-male specific RNA phage, does not multiply in water below 25 °C, but excluded the host in the exponential growth phase. It might be that in raw and biologically treated sewage these indicators do multiply and consequently alter the expected correlation between the levels of the phages and enteric viruses.

Unquestionably, improvement in bacteriophage detection and host specificity will result in a better correlation between their levels and those of various pathogens present in polluted water. However, despite our recent and future progress, we should narrow the definitions of indicators according to the degree of pollution and its presence. In brief, the bacteriophage groups that have been related to a certain pollution criteria and found to correlate

(continued)

with the presence of human pathogens do not need to correlate with the same pathogen in a different pollution environment or in a different geographical area. The ubiquity that we seek so intensively in order to adapt it for guidelines is perhaps the main pitfall in reaching the right conclusions. An excellent example for the above assumptions is the *B. fragilis* bacteriophage group: the bacterial hosts used in Spain for their specific phages is *B. fragilis* HSP-40, resulting in good phage detection from human fecal wastes, while *B. fragilis* RYC2056 detects phages in both human and animal fecal wastes (Puig et al. 1999). For example, on the global scale, the *B. fragilis* RYC2056 host showed good results, while *B. fragilis* HSP40 showed geographic variability, as mentioned earlier (high counts for Southern Europe, Israel, South Africa versus low counts for the USA, Sweden, and the UK). Chung et al. (1998) showed that *B. fragilis* VPI3625 used in USA was similar in its plaquing efficiency to *B. fragilis* RYC2056.

In summary, there are several critical issues that still need clarification before the introduction of bacteriophages as routine indicators of viral pollution of water resources. These are: (1) detection methodology, that is choosing the host and phage choice according to water source, geographical site, and past experience (Furuse et al. 1983); (2) validation methods based on inter- and intra-laboratory reproducibility (mainly at the country level); (3) establishment of specificity; (4) sensitivity increase through selection; (5) epidemiological support by combined studies including enteric viruses; and (6) low cost and simplicity of routine tests performed by water laboratories. Finally, a recent publication theoretically suggested the use of the Torque teno virus transmitted primarily via the fecal-oral route in humans (based on the presumption that this enteric virus is “ubiquitous in humans, elicits seemingly innocuous infections, and does not exhibit seasonal fluctuations or epidemic spikes”) as an appropriate indicator of viral contamination of drinking water (Griffin et al. 2008). Still, this theoretical proposition preeminently emphasizes the major indicator challenge: (1) it needs to be tested, including in terms of densities and occurrences, including spatial and temporal stability; (2) a viral assay and infectivity test has yet to be developed; and (3) determination and correlation along real enteric pathogens!

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