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A REVIEW

Bacteriophages as indicators of enteric viruses and public health risk in groundwaters

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1. INTRODUCTION

For more than 100 years, there has been debate among microbiologists and public health practitioners concerning the role of drinking water monitoring for either pathogens or indicators. By the 1890s, it was generally accepted that monitoring for specific pathogens was not in the best interest for public health protection because there were too many pathogens, they were present in very small concentrations, and methods for their detection were not practical. Public health officials decided that monitoring would be conducted to detect faecal pollution rather than individual pathogens.

Escherichia coli became the primary indicator of faecal pollution at that time. As methods for the specific detection and identification of *E. coli* were not well developed, an *E. coli* surrogate — the total coliform group — was developed. Its detection was based on the physiological observation that the fermentation of lactose in a growth medium, which allowed the manifestation of only enteric bacteria, would elucidate *E. coli* and its relatives, the total coliforms. Shortly afterwards, coliform monitoring became codified throughout the world. Coliform monitoring, being rapid, inexpensive, and easy to perform, proved effective. The rates of water-borne bacterial disease outbreaks in developed countries today are low.

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In the 1970s, the role of drinking water in the transmission of enteric viruses became a subject for concerted studies. It was found that coliforms might differ biologically from the fate of animal viruses in drinking water (Kraus 1977; Berg 1978; Berg and Metcalf 1978; Engelbrecht and Greening 1978; Scarpino 1978; Gerba *et al*. 1979; Olivieri 1982; Pipes 1982; Block 1983; Farrah and Schaub 1983; Goyal 1983; Vaughn and Landry 1983; Stetler 1984; Borrego *et al*. 1987; Kator and Rhodes 1994; Leclerc 1997).

Overall, the conclusion with regard to monitoring for enteric viruses is much the same as that decided by public health officials in the 1890s. To protect the public's health, it is better to monitor for the indicators of faecal pollution than for specific pathogens. The over-riding rationale for this conclusion is that there are still unknown (and simply, too many) enteric viral pathogens. Moreover, they are present in low concentrations, often requiring the analysis of more than 10 l of water. Although methods have been proposed for many of the enteric viruses, they are generally expensive, technically demanding and time-consuming. For distribution water, where the strategy is to detect relatively recent faecal contamination events, *E. coli* and total coliforms are the most useful because they can be monitored frequently and inexpensively with simple, specific tests (Edberg *et al*. 1988, 1989).

For groundwater, while this strategy has proved effective, the situation is compounded. Here, the infectious particle

must travel throughout the aquifer or subterranean water source. As viruses are smaller than bacteria, there has been concern that the transit times of the bacterial indicators may not be equivalent to those of the human enteric viruses. Moreover, there is evidence that some viral pathogens may be differentially resistant to environmental conditions, sewage or water treatment processes, compared with coliform organisms (Clarke and Kabler 1954; Shuval 1970, 1976; Shuval *et al.* 1971; Englebrecht *et al.* 1974; Katzenelson *et al.* 1974; Wellings *et al.* 1975; Bates *et al.* 1978; Berg *et al.* 1978; Marzouk *et al.* 1980; Payment *et al.* 1985, 1993; Jofre *et al.* 1995; Gantzer *et al.* 1998). There are circumstances in which viruses may survive in the water environment when indicator faecal bacteria do not (Wellings *et al.* 1975; Shaffer *et al.* 1980; Hejkal *et al.* 1982; Rose *et al.* 1986; Craun 1992; Metcalf *et al.* 1995). Accordingly, with recent developments in the ability to detect enteric viral pathogens and viral surrogates, it is appropriate to consider how best to monitor for enteric viruses in groundwater. This review will consider both direct detection methods and, primarily, the proposed viral surrogate and faecal indicator, the bacteriophages.

2. DIRECT VIRUS DETECTION

There are more than 140 enteric viruses identified from human faeces. However, at concentrations found in sewage, a limited number appears able to produce gastroenteritis. Most important are the Hepatitis A and E viruses (HAV/HEV), caliciviruses (including Norwalk virus), rotaviruses and astroviruses. Methods for detection of these viruses are making excellent progress. However, at this time, they are not amenable to routine use for drinking water. Viruses can be elucidated either by cell culture or by molecular biology methods, primarily relying on genetic amplification or combinations. Generally, cell culture is expensive, requires a highly specialized laboratory, is cell-line specific (certain cell lines will recover only certain virus types) and requires confirmatory tests. Genetic amplification of viral nucleic acid is the most rapidly growing area of detection. Genetic amplification is expensive, restricted to specialized laboratories, highly subjective to contamination and requires confirmatory steps. Moreover, each molecular probe set will only detect a single virus. Most importantly, genetic amplification techniques do not currently have the ability to differentiate living from inactive, or non-infectious, viruses. Large sample volumes (of the order between 10 and 100 l) need to be processed for enteric viruses. Such large volumes virtually preclude the shipping of samples to central laboratories. Therefore, the use of these pathogen detection methods for the monitoring of drinking water and groundwater is premature. Their major use currently lies in identifying the specific viruses that may be present in a groundwater source in a research protocol.

It has been suggested that pan-enterovirus probes, general indicators of enteric viral pathogens, should be used in direct tests for HAV, caliciviruses, rotaviruses and astroviruses (Deleon *et al.* 1990; Margolin *et al.* 1991; Schwab *et al.* 1991, 1993). However, there has been little consistent correlation between enteroviruses and HAV occurrence (Dubrou *et al.* 1991; Shieh *et al.* 1991; Leguyader *et al.* 1993, 1994; Tsai *et al.* 1993), and other groups of enteric viruses may show different survival and disinfection-resistant behaviour (Block 1983; Hoff and Akin 1983; Schwartzbrod 1991; West 1991). Therefore, the value of enteroviral detection in predicting a potential public health hazard of viral disease is no better, and may be worse, than that of a faecal bacterial indicator such as *E. coli* or Enterococci (Metcalf *et al.* 1995).

The development of molecular methods has facilitated the detection of viruses that replicate poorly, or not at all, in cell cultures. Several studies were carried out using the polymerase chain reaction (PCR) to detect human viruses, including enteroviruses, adenoviruses and Hepatitis A viruses, in the environment and in shellfish samples (Girones *et al.* 1993, 1995; Puig *et al.* 1994; Tani *et al.* 1995; Pina *et al.* 1998). More recently, Pina *et al.* (1998) suggested that the detection of adenoviruses by PCR could be a better molecular index of the presence of human viruses in the environment than enteroviruses. Moreover, adenoviruses are more stable in various environmental conditions than enteroviruses, and are more resistant to ultraviolet irradiation and biocide agents (Irving and Smith 1981; Sobsey *et al.* 1986; Enriquez *et al.* 1995; Meng and Gerba 1996). As with all genetic amplification methods for viruses at this time, however, the assay does not provide viability information.

There is much work being done to make the genetic amplification methods practically useful. Investigators are working on a multiplex PCR, which allows a simultaneous amplification of many different viruses. They are also examining the means by which viability parameters may be identified, attempting to compartmentalize the amplification so as to avoid cross-contamination of the laboratory, and combining cell culture with genetic amplification for both assay viability and identification (Gantzer *et al.* 1998).

3. BACTERIOPHAGES

Bacteriophages are attractive candidates for indicators of enteric viruses in groundwater. Foremost, they are viruses themselves. Bacteriophages arise from host bacteria that become infected and subsequently lyse. Some groups of bacteriophages (or 'phages') are able to infect members of the normal bacterial flora of the human gastrointestinal tract and are found in sewage and wastewater, but rarely in human faeces. Bacteriophage assay conditions are much simpler and cheaper than any of the enteric virus detection methods. In order to be suitable enteric viral indicators for groundwater,

bacteriophages should have characteristics which include: specificity, i.e. they occur consistently and exclusively in human faeces and sewage, do not multiply in the environment and do not have a faecal environmental source; sensitivity, i.e. they are present in greater numbers than the enteric viruses; they are at least as long-lived as the enteric viruses present in the environment and throughout the water treatment processes; their detection must be easy to perform by simple and inexpensive methods; and their characteristics in the aquifer should closely mimic those of the enteroviruses (Table 1) (Keswick *et al.* 1984; Payment and Franco 1993; Hurst *et al.* 1994).

Three basic groups of bacteriophages have been proposed as candidate indicators for the enteric viruses: somatic coliphages (Kott 1966; Hilton and Stotzky 1973; Kott *et al.* 1974; Iawprc 1991), male-specific RNA coliphages or FRNA phages (Havelaar and Hogeboom 1984; Iawprc 1991) and phages infecting *Bacteroides fragilis* (Armon and Kott *et al.* 1993; Jofre *et al.* 1986; Tartera and Jofre 1987; Iawprc 1991; Grabow *et al.* 1995; Gantzer *et al.* 1998a). The phage replication cycle begins when the bacteriophage adsorbs to the bacterial host at specific cell-surface sites, e.g. particular components of the cell wall (somatic phages) the flagellum (flagellotropic phages), a sex pilus (male or FRNA phages) etc. Therefore, somatic phages attach to the body of the bacterium at the cell membrane or cell wall. FRNA phages attach to the F pili of the cells and are therefore referred to as F^+ phages. The bacteriophages that infect *E. coli* and other coliforms, as well as possibly other members of the Enterobacteriaceae, are referred to as coliphages. For phage development to proceed, the phage nucleic acid must penetrate the cell wall and escape the degradation by the various nucleic endonucleases of the host cell (Table 2).

One of the problems with using the presence of coliphages as indicators of faecal contamination and enteric viruses is their lack of specificity for the bacterial host. For example, while F^+ coliphages are generally specific for *Escherichia coli*, they may also attack and multiply in other coliforms and Enterobacteriaceae (Bradley 1967; Dhillon *et al.* 1970; Dhillon and Dhillon 1974; Goyal 1987; Rhodes and Kator 1991; Ackerman, *et al.* 1992). Also, the male host cell can undergo FDNA coliphage attack, as well as somatic coliphage attack (Qureshi and Qureshi 1991; Qureshi *et al.* 1988; Debartolomeis and Cabelli 1991; Rhodes and Kator 1991; Payment and Franco 1993; Williams and Stetler 1994; Hsu *et al.* 1996; Sinton *et al.* 1996; Stetler and Williams 1996). Accordingly, because they are not specific to the *E. coli* host and are not normally found in the faeces of humans, they cannot be good indicators of the presence of faecal contamination and enteric viruses (Furuse *et al.* 1983; Havelaar *et al.* 1985, 1986, 1990; Furuse 1987). They have been found in waters free of faecal pollution and are suspected of replicating in water (Seeley and Primrose 1980; Rhodes and Kator 1991). Conversely, they have not been detected in waters high in faecal pollution (Qureshi and Qureshi 1991; Rhodes and Kator 1991). In addition, cooler temperatures can limit their replication in wastewater and groundwater (Woody and Oliver 1995). These contradictions make them unreliable as indicators of faecal contamination and enteric viruses.

Somatic coliphages, which are a more heterogenous group, are generally not considered to be good faecal indicators as their hosts are unlikely to be of faecal origin (Hsu *et al.* 1996). There are many non-faecal sources of coliform bacteria. Accordingly, it is difficult to ascertain specifically whether an isolate of a somatic coliphage arose from any one point of contamination. Coliforms, which can colonize biofilms, may be present anywhere in the water collection treatment or distribution system. Multiplication of F^+ coliphages and

Table 1 Factors that may influence virus movement to groundwater

Factor	Comments
Soil type	Fine-textured soils retain viruses more effectively than light-textured soils. Iron oxides increase the adsorptive capacity of soils. Muck soils are generally poor adsorbents.
pH	Generally, adsorption increases when pH decreases. However, the reported trends are not clear-cut due to complicating factors.
Cations	Adsorption increases in the presence of cations (cations help reduce repulsive forces on both virus and soil particles). Rain water may desorb viruses from soil due to its low conductivity.
Soluble organics	Generally compete with viruses for adsorption sites. No significant competition at concentrations found in wastewater effluents. Humic and fulvic acid reduce virus adsorption to soils.
Virus type	Adsorption to soils varies with virus type and strain. Viruses may have different isoelectric points.
Flow rate	The higher the flow rate, the lower virus adsorption to soils.
Saturated *vs* unsaturated flow	Virus movement is less under unsaturated flow conditions.

Taken from Gerba and Bitton (1984).

Table 2 Major groups of indicator bacteriophages

Group	Family	Nucleic acid	Tail type	Host cell attack	Bacteriophages	nm
A	Myoviridae	Dbl strand DNA	Contractile	Somatic	T2, T4, T6 (T even phages)	95 × 65
B	Siphoviridae	Dbl strand DNA	Long, non-contractile	Somatic	λ, T5	54
C	Podoviridae	Dbl strand DNA	Short non-contratile	Somatic	T7, T3	47
D	Microviridae	Sngl strand DNA	No tail, large capsomeres	Somatic	ϕX174, S13	30
E	Leviviridae	Sngl strand DNA	No tail, small capsomeres	F^+ pili	Group 1: MS-2, f2, R-17, JP501 Group 2: GA, DS, TH1, BZ13, KU1, JP34 Group 3: Qβ, VK, ST, TW18 Group 4: SP, FI, TW19, TW28, MX1, ID2	20–30
F	Inoviridae	Sngl strand DNA	No head, flexible filament	F^+ Pili	SJ2, fd, AE2, M13	810 × 6

References: Bradley 1967; Tomoeda *et al.* 1975; Furuse, *et al.* 1979; Sobsey *et al.* 1995.

somatic coliphages in environmental coliforms can lead to a false-positive indicator analysis.

Moreover, it should be noted that phages are, in fact, indicators of indicators. Somatic coliphages arise from total coliforms (including *E. coli*) and F^+ phages arise from 'male specific' *E. coli* cells and other total coliforms. Both may also arise from other Enterobacteriaceae. Therefore, phages are not true indicators of faecal contamination and enteric viral contamination. They resemble enteric viruses only in that they are resistant to disinfection, are of the same approximate size (except for the somatic and FDNA phages) and have similar movement through soils. One of the major discussions and points of controversy concerning the use of coliphages as indicators of faecal contamination is the fact that the parent indicator (*E. coli* or total coliforms) must be present in the aquifer and in sufficient concentration (10^4 ml^{-1}) to promote bacteriophage replication (Wiggins and Alexander 1985). With the low temperatures found in groundwater, especially in temperate regions, the necessary density of the host would be difficult to achieve (Woody and Cliver 1995). Also, because of the colder temperatures, F^+ pili production would be severely limited (Novotny and Lavin 1971; Tomoeda *et al.* 1975; Seeley and Primrose 1980). The multiplicity of hosts in use in these studies further compounds the problem (Table 5). It is important that the bacterial hosts chosen for the phage assay are: (i) easy to work with; (ii) stable; (iii) preferably possessing antibiotic resistance; and (iv) not harbouring lysogenic phages which can activate unexpectedly and result in false-positive tests. For these reasons, many public health practitioners feel that *E. coli* (for which there are rapid, simple, inexpensive methods currently available) is still the best faecal indicator overall. Conversely, coliphage monitoring over the last 30 years has contributed little to public health protection.

While it is well established that the sample volume for bacterial indicators is 100 ml, most work with bacteriophages has been carried out on 15 l. It is generally accepted that this large volume of sample is not practical for routine monitoring. Considerable work is now being conducted in an attempt to increase the efficiency of phage capture from water samples so that smaller volumes may be taken. In particular, it is important to absorb the target phage from the water sample differentially in order to concentrate it. The process of absorption should not be so harsh as to render the phage injured or non-viable.

3.1 Somatic coliphages

Guelin (1948) was the first to advocate the use of coliphages as indicators of enteric virus pollution. In the 1970s and 1980s, a number of studies explored the use of coliphages as alternative indicators of enteric viruses in wastewater and other faecally-contaminated waters (Buras and Kott 1966; Coetzee 1967; El Abagy *et al.* 1988; Kott et al. 1974, 1978; Kott 1977a, 1981; Hilton and Stotzky 1973; Scarpino 1975; Grabow *et al.* 1984; Stetler 1984; Wiggins 1985; Havelaar *et al.* 1986). A large number of coliphage–host systems has been investigated and work is continuing in the field to examine others (Dhillon *et al.* 1970, 1976; Seeley and Primrose 1979, 1980; Osawa *et al.* 1981; Primrose *et al.* 1982; Furuse *et al.* 1983; Havelaar and Hogeboom 1983, 1984; Havelaar *et al.* 1985, 1986; Furuse *et al.* 1987; Gerba 1987; Goyal 1987; Havelaar and Pot-Hogeboom 1988; Qureshi *et al.* 1988; Ketratanakul and Ohgaki 1989; Havelaar *et al.* 1990; Debartolomeis and Cabelli 1991; Karst *et al.* 1991; Kfir *et al.* 1991; Rhodes and Kator 1991; Armon and Kott 1993; Payment and Franco 1993; Ijzerman *et al.* 1994; Williams and Stetler 1994; Sobsey *et al.* 1995; Woody and Oliver 1995; Hsu *et al.* 1996;

Sinton *et al.* 1996; Stetler and Williams 1996; Araujo *et al.* 1997; Gantzer *et al.* 1998a; Hsu *et al.* 1998; Muniesa and Jofre 1998). Most commonly, *E. coli* B, C, CN, CN13, C-3000, F-amp, and K-12 derivatives such as WG21 and W3110 plus several undesignated strains of *E. coli*, and *Salmonella typhimurium* strains WG45 and WG49 (with the added *E. coli* F-plasmid responsible for F pili production), have been used (Table 5).

Somatic coliphages detected by these commonly used host strains are a heterogeneous group with different characteristics but the same basic receptor site, which occurs on the cell wall. Scarpino (1978) described some of these coliphages (such as the T1 through T7 double-stranded DNA phages), their morphology, their bacterial hosts, their burst sizes, and other features. Bradley (1967), Furuse *et al.* (1979), Furuse (1987) and Sobsey *et al.* (1995) described in greater detail the groups of somatic, FRNA, FDNA bacteriophages. Kott and colleagues expanded the observations of Scarpino to determine whether coliphages were valid indicators of enteric viral pollution. They found that the phages were present in wastewater and other faecally-contaminated waters in numbers at least equal to the enteric viruses (Fannin *et al.* 1977; Kott 1977b; Kott, *et al.* 1969, 1974, 1978; Longley 1978), they persist in wastewaters, surface waters and sand columns for longer than enteroviruses (Kott 1977a, 1977b; Kott *et al.* 1969, 1974, 1978),), and they are detected by available methods (Kott *et al.* 1974; Grabow *et al.* 1978). Coliphages have also been suggested as indicators of viruses in aerosols emitted from wastewater treatment plants, as they are more stable than coliforms in an air-borne state (Fannin *et al.* 1977).

Comparative studies by Vaughn and Metcalf (1975) on coliphages and enteric viruses in sewage effluents, shellfish and shellfish-growing waters during a period of 3 years led to the suggestion that coliphages have serious shortcomings as indicators of enteric viruses. These concerns and limitations centred on several factors: the replication of coliphages and the presence of more than one dominant phage type in estuarine and fresh waters (Vaughn and Metcalf 1975; Seeley and Primrose 1980; Parry *et al.* 1981; Borrego *et al.* 1990); no correlation between the densities of coliphages and enteroviruses in raw sewage and farm pond water (Joyce and Weiser 1967; Safferman and Morris 1976; Havelaar *et al.* 1987; Nieuwstadt *et al.* 1991; Wommack *et al.* 1996); inconsistent occurrence of coliphages in raw sewage samples with the simultaneous isolation of few enteroviruses and, by contrast, a high number of enteroviruses isolated in many treated effluents with no coliphages detected (Vaughn and Metcalf 1975); the inability of some 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); and host-strain variability (Havelaar *et al.* 1986). Justification for the use of coliphages as sentinels of enteric viruses in wastewater and other faecally-contaminated waters has been reviewed by Gerba (1987), the IAWPRC study group on Health Related Water Microbiology (IAWPRC 1991) and Limsawat and Ohgaki (1997).

Somatic coliphages suffer from another potentially important but critical limitation. They are not specific to *E. coli*. There is evidence that somatic coliphages may multiply in species of Enterobacteriaceae which are part of the total coliform group and often found associated with vegetation and biofilms. Of these, the two most common species are *Klebsiella pneuomoniae* and *Enterobacter cloacae*. Therefore, it is possible that coliphages might be produced which are not only unrelated to faecal contamination but also,unrelated to any health risk. Accordingly, the use of somatic coliphages as indicators of faecal pollution and enteric viruses has serious shortcomings.

3.2 F^+-Specific bacteriophages

There are two groups of F^+-specific bacteriophages, Group E and Group F, out of a total of six bacteriophage groups (Singleton and Sainsbury 1993). The first group, Group E (Leviviridae), comprises four sub-groups (I-IV). Group E phages have small, hexagonal capsomeres without tails. They are approximately 20–30 nm, containing single-stranded RNA. These are referred to as the FRNA coliphages. They have been classified into four groups (I, II, III and IV) on the basis of serological and physicochemical properties. These FRNA coliphages include MS-2 in group I, GA in group II, Q in group III and SP in group IV. These phages are relatively resistant to disinfectants, sunlight, heat treatment and water- and sewage-treatment processes (Havelaar and Hogeboom 1984; Havelaar and Niewstad 1985). Group III has been the most intensively studied. The FRNA coliphages are infectious to bacteria which possess the F-plasmid, and this F-plasmid is transferable to a wide range of Gram-negative bacteria (Sobsey *et al.* 1995). This transferability raises concern over the lack of *E. coli* specificity, since there is evidence to suggest that environmental coliphages may assume this plasmid (Dhillon and Dhillon 1974; Havelaar *et al.* 1985; Havelaar and Pot-Hogeboom 1988). In fact, plasmids have been shown to be transferred among species of coliforms, including *E. coli* and *Salmonella, Shigella,* and *Bacteroides* (Shaw and Cabelli 1980; Corliss *et al.* 1981).

The second group, Group F (Inoviridae), is made up of large, filamentous bacteriophages which are 760–1950 × 6 nm in size. They have no head and consist of a flexible filament containing single-strand DNA. These are referred to as the FDNA coliphages. Both the FRNA and FDNA bacteriophages have been found in wastewater, but the sanitary significance of FDNA phages has not been determined (Sinton *et al.* 1996). FRNA phages have been detected in raw sewage at 1000 pfu ml^{-1} (Furuse 1987; Havelaar *et al.* 1990,

1993; IAWPRC 1991), but FRNA and FDNA phages are rarely found in human faeces. Table 3 presents the densities of F^+, or male-specific, bacteriophages in human-associated wastewaters.

The predominating FDNA coliphages have received less attention as indicators of enteric viruses because they are generally less plentiful than FRNA coliphages. They do not resemble human enteric viruses morphologically, and their ecology is poorly understood. The infectious process of FRNA coliphages can be inhibited by the presence of RNase in the growth medium, which can be used to determine whether FRNA coliphage attack has occurred. Unfortunately, the FRNA phages have been shown to have resistance to RNase. The order of resistance determined by Havelaar (1986) for the FRNA phage groups are as follows: IV > III > I > II, group IV being the most resistant. In this case, because of a lack of specificity even with the FRNA coliphages, there is no simple way of confirming the presence of FRNA phages.

The *E. coli* male-specific coliphages are primarily produced in *E. coli*, which is carried in the intestinal tract of all mammals. However, unlike the parent bacterium, which is present in all mammals at extremely high concentrations in the range of 10^9 g^{-1}, the F^+ coliphages are present in much lower concentrations and can vary by species (Table 4). There is also evidence that the F^+ phages may reproduce in members of the total coliform group (other than Enterobacteriaceae), thus raising specificity concerns.

Moreover, in a single individual, the F^+ phages may be absent. It has been reported that only approximately 3% of humans carry the F^+ phages in their *E. coli*. Therefore, it is of concern among public health microbiologists that the concentrations of F^+ coliphages may not be high enough to provide sufficient public health protection as a monitoring tool (Havelaar *et al.* 1990, 1993; Debartolomeis and Cabelli 1991;Woody and Oliver 1995). In addition to a lack of specificity, there is a lack of sensitivity.

There is a reported variability about the methods used to detect and analyse F^+ coliphages. Most outstanding are concerns regarding candidate bacterial hosts. Some host strains of bacteria are reported to be permissive (restrictive) only to F^+ phage replication, whereas others are permissive for both FRNA and somatic coliphages. Havelaar and Hogeboom (1984) and Debartolomeis and Cabelli (1991) have reported the development of FRNA coliphage hosts that were selected for their resistance to somatic coliphages, as well as antibiotic resistance, to minimize overgrowth by naturally occurring bacterial flora in environmental samples. In reality, all FRNA phage hosts currently in use are restrictive not only to FRNA phages. The Havelaar host strain was constructed by inserting an F^+-plasmid (F'42 lac: Tn5) into a strain of *Salmonella typhimurium*, designated WG49. This clone apparently lacks the cell wall receptors for somatic coliphages but frequently undergoes attack from somatic *Salmonella* phages. The DeBartolomeis and Cabelli host, designated *E. coli* F-Amp, is resistant to both ampicillin and streptomycin and has been chosen by the EPA for the Institute for Cooperative Research (ICR). Another host strain, designated *E. coli* C3000, was proposed by the US EPA ICR (IAWPRC 1991) for information collection monitoring in water supplies but was withdrawn, probably because of infection by somatic coliphages (Hsu *et al.* 1996). *Escherichia coli* C3000 has sex-pili which allow for the replication of F^+-specific and somatic coliphages. C3000 is not resistant to antibiotics, may become contaminated with naturally occurring bacteria in surface waters, and has been known to show somatic cell attack, primarily.

Table 3 Densities of male-specific bacteriophage (MSB) in human-associated wastewaters

		MSB density*			
Waste type	No. of samples	By qualitative enrichment method (%)	By quantitative direct-plating method (%)	Range	Mean MSB†
Human faeces	13	1 (8)	0·0 (0)	$<1{\cdot}0$–$6{\cdot}25$	<1
Residential lift station	22	—‡	2 (9)	$<50{\cdot}0$–$2{\cdot}5 \times 10^4$	$1{\cdot}3 \times 10^3$
Sewage plant effluent	14	—	11 (79)	<100–$2{\cdot}1 \times 10^5$	$3{\cdot}0 \times 10^4$
Septage	17	11 (65)	10 (59)	<10–$1{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^5$
In-line sewage	11	—	11 (100)	$4{\cdot}5 \times 10^3$–$8{\cdot}7 \times 10^5$	$2{\cdot}3 \times 10^5$
Sewage plant influent	14	—	14 (100)	$8{\cdot}5 \times 10^4$–$3{\cdot}4 \times 10^6$	$5{\cdot}2 \times 10^5$

* pfu g^{-1} faeces or 100 ml^{-1} sewage. Range was determined by the direct-plating procedure.
† Arithmetic mean MSB values were determined from quantitative direct-plating results.
‡ —, Not determined.
Taken from Calci *et al.* (1998).

Table 4 Daily per capita MSB (Male Specific Bacteriophage) loadings for different animal species and a hypothetical 1 million gallons d^{-1} wastewater treatment plant, calculated from mean MSB densities determined for animal faeces and wastewaters

Waste source or type	Mean MSB density (pfu g^{-1})*	Excrement generated (g d^{-1})†	Estimated MSB released into the environment (pfu d^{-1})
Source			
Canine	2·1	412·5	$8{\cdot}6 \times 10^2$
Sheep	1·5	1130·0	$1{\cdot}7 \times 10^3$
Goat	1·9	1200·0	$2{\cdot}3 \times 10^3$
Duck	13·1	336·0	$4{\cdot}4 \times 10^3$
Goose	39·2	500·0	$2{\cdot}0 \times 10^4$
Steer	4·7	23 600·0	$1{\cdot}1 \times 10^5$
Chicken	1867·0	182·3	$3{\cdot}4 \times 10^5$
Bay seagull	888·2	500·0	$4{\cdot}4 \times 10^5$
Dairy cow	84·3	23 600·0	$2{\cdot}0 \times 10^5$
Landfill seagull	11 848·8	500·0	$5{\cdot}9 \times 10^6$
Hog	4136·5	2700·0	$1{\cdot}1 \times 10^7$
Horse	950·2	20 000·0	$1{\cdot}9 \times 10^7$
Type			
Septage ($n = 17$)	1000·0	$1{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^9$
Sewage effluent ($n = 14$)	300·0	$3{\cdot}8 \times 10^9$	$1{\cdot}1 \times 10^{12}$
Sewage effluent ($n = 14$)	5200·0	$3{\cdot}8 \times 10^9$	$2{\cdot}0 \times 10^{13}$

* For types of waste, mean MSB density is expressed as pfu ml^{-1}.
† For types of waste, excrement generated is expressed as ml d^{-1}. Taken from Calci, *et al.* 1998.

Havelaar and Hogeboom (1984) developed a method for the direct enumeration of FRNA phages in faeces and fresh water, based on the specially constructed host strain, *Salmonella typhimurium* WG49. They reported that the FRNA phages were infrequently found in human and animal faeces but were consistently isolated in sewage in concentrations ranging between 10^2 and 5×10^4 pfu ml^{-1} (Osawa *et al.* 1981b; Furuse *et al.* 1983a,b; Havelaar *et al.* 1986, 1990). The reasons for the difference in isolation between humans and the higher numbers found in sewage have not been resolved (Beekwilder *et al.* 1996). It is postulated that the lower temperature in sewage may permit more efficient phage multiplication. The four major groups (I, II, III and IV) are found at different prevalences in human and animal sources and different geographical distributions (Snowdon and Cliver 1989). Therefore, at this time, FRNA coliphages can be said to be consistently present, in the 1000–10 000 pfu l^{-1} range, in sewage arising from large numbers of people (Beekwilder *et al.* 1996).

From sources other than large-volume sewage, such as groundwater, the significance of FRNA coliphages as sentinels of enteric viruses remains to be established. Several issues of concern must be resolved. First, there is no consensus on a bacterial host. Assay interference due to the presence of somatic *Salmonella* (SS) phages has been reported in the examination of surface waters (Rhodes and Kator 1991; Handzel *et al.* 1993). In the study by Rhodes and Kator (1991), 99% (293 of 294) of the total number of plaques produced on *Salm. typhimurium* WG49 were produced by RNase-resistant phages. As strain WG49 is naturally susceptible to SS phages, such assay interference is a continuing problem in waters where SS phages outnumber FRNA coliphages. It is not uncommon for SS phages to occur in greater numbers than FRNA coliphages in surface waters (Rhodes and Kator 1991; Handzel *et al.* 1993) and in vulnerable groundwater (Williams and Stetler 1994; Stetler and Williams 1996). The common occurrence of SS phages at higher levels than FRNA coliphages compromises the usefulness of the WG49-based assay, and therefore, a method more specific for FRNA phages is desirable (Handzel *et al.* 1993; Stetler and Williams 1996). Moreover, because of the occurrence of interfering FDNA phages as well, it is necessary to perform parallel assays without RNase testing, thus increasing the cost and complexity of the assay. Also, as discussed earlier, FRNA phage groups have been shown to be resistant to RNase.

In view of the generally low level concentrations of FRNA

Table 5 Indicator coliphage hosts

Host	Strain	Reference	Strengths	Weaknesses	Stability
S. typhimurium (F^-)	WG45[1]	1, 8, 10, 1, 24, 27, 31, 32, 42	Detects somatic Salmonella phages.	Shows only somatic attack.	
S. typhimurium (F^+)	WG49[2]	1, 2, 3, 8, 10, 16, 24, 27, 31, 32, 36, 42	When sampling sewage samples, reported to be selective for F-RNA phages. Gave higher counts than WG61 Plasmid free strain. Low rate of F^- plasmid segregation (1). Kanamycin & nalidixic acid resistant (3).	Not specific to F-RNA phages: also susceptible to attack by salmonella somatic phages and F-DNA phages. Somatic salmonella phages caused major interference (8, 10, 16, 32). Development of an *E. coli* host is preferable (39).	An unstable strain and unpredictability loses its ability to plaque F-specific phages (16).
S. typhimurium (F^+)	SA2197[3]	1	Difference from other strains, F plasmid carries the *fin*-mutation and has a low segregation rate.	Not specific to F-RNA phages: infected by F^+ Salmonella & F^+ *E. coli* phages, as well as F^- *E. coli* phages. Produced thin bacterial lawns. Plaques difficult to count.	
S. indiana (F^+)	WG61[4]	1, 3	Clearer plaques with MS2.	WG61 counts not as high as with WG49. Produces a bacteriocin (1).	
E. coli (F^-)	CN[5], CN13	1, 5, 24, 32, 34, 37, 43, 44	Nalidixic acid resistant strain.	Shows only somatic attack.	
E. coli (F^-)	K-12[12]	1, 2, 5, 25, 26, 35		Shows only somatic attack.	
E. coli (K-12 F^+)	WG21[6], A/λ, Q13	1, 3, 5, 12, 13, 15, 20, 24, 26, 27, 35, 47		Susceptible to F-DNA phage attack (35). Also, plaque somatic T phages (39). Highly inefficient for enumeration of naturally occurring FRNA phages (3).	
E. coli (F^-)	B[7]	1, 5, 6, 11, 19, 24, 26, 35, 39		Produced plaque counts 5–6 times lower (49). Also, plaque somatic T phages (39).	
E. coli (F^-)	C[8]	5, 6, 8, 11, 13, 18, 19, 24, 27, 35, 37, 39, 47	More plaques, highest counts (5). Nalidixic acid resistant. Most suitable for isolating DNA somatic phages, especially temperate phages (19, 48).	Isolates also infectious to *S. sonnei* (wild type) (5). Also, plaque somatic T phages (39).	
E. coli (F^+)	C-3000[10]	8, 16, 31, 36, EPA ICR Proposed, 42		May be infected by some somatic coliphages. Majority of phages were somatic (36).	
E. coli (K-12 F^+)	W3110[9]	12, 13, 33, 35, 38, 47			
E. coli	R AMP[11], RR[13]	16, 36, 37, 39, EPA ICR, 43, 44	Ampicillin & Streptomycin resistant. Gave the highest % of detection for FRNA phages (36). Greater specificity for surface waters.	Low counts & susceptible to FDNA phage attack (16, 36).	*E. coli* RR, stable (42).

[1]In reference 1: mutant of WG42; also, references 8, 16, 27. [2]In reference 1: mating of WG45xWG27; also, references 8, 16, 27, 32, 36. [3]Reference 1. [4]Reference 1. [5]Reference 1, 5, 43. [6]References 20, 35, 26, 27. [7]Reference 26. [8]Reference 16. [9]Reference 35. [10]Reference 16. [11]Reference 39; also references 16, 36. [12]Reference 26. [13]Reference 42. References: 1 = Havelaar & Hogeboom 1984; 2 = Havelaar and Pot-Hogeboom (1988b); 3 = Havelaar *et al.* 1985; 5 = Havelaar and Hogeboom 1983; 6 = Qureshi and Qureshi 1991; 8 = Williams and Stetler 1994; 10 = Stetler and Williams 1996; 11 = Karst *et al.* 1991; 12 = Havellar, *et al.* 1990; 13 = Seeley and Primrose 1980; 15 = Woody and Oliver 1995; 16 = Rhodes and Kator 1991; 18 = Gantzer *et al.* 1998; 19 = Furuse *et al.* 1983; 20 = Ketratanukul & Ohgaki 1989; 24 = Havelaar *et al.* 1986; 25 = Dhillon *et al.* 1976; 26 = Dhillon *et al.* 1970; 27 = Kfir *et al.* 1991; 31 = Sobsey *et al.* 1995; 32 = Payment and Franco 1993; 33 = Seeley and Primrose 1979; 34 = Muniesa and Jofre 1998; 35 = Qureshi *et al.* 1988 36 = Hsu *et al.* 1996; 37 = Hsu *et al.* 1998; 38 = Osawa *et al.* 1981a; 39 = Debartolomeis and Cabelli 1991; 42 = Sinton *et al.* 1996; 43 = Araujo *et al.* 1997; 44 = Armon and Kott 1993; 47 = Primrose, *et al.* 1982.

phages, particularly in groundwater, large sample volumes need to be assayed. Most commonly, double-layer agar methods have been used. Stetler and Williams (1996) analysed 58 samples and found an average FRNA coliphage concentration of 140 pfu $100\,l^{-1}$ of groundwater. Therefore, public health practitioners may be required to sample volumes of at least 1 litre and up to 100 l. Such large-volume sampling requires the use of concentration methods, which were generally developed for human enteric viruses. Large-volume sampling with a concentration step is an extremely critical area of research and is severely limiting for broad-scale application to bacteriophage testing.

The use of direct pour plate assay methods for phage enumerations generally requires a number of steps to reduce or suppress the growth of naturally occurring micro-organisms. Incorporation of antibiotics or decontamination with chloroform (Osawa *et al.* 1981a; Tartera and Jofre 1987), membrane filtration (Tartera and Jofre 1987) and selective media (Kennedy *et al.* 1985) have been used for this purpose. The combined use of membrane filtration with incorporation of the antibiotic, nalidixic acid, has been used most commonly to increase overall assay selectivity.

Bacteriophages are most consistently associated with large-volume sewage and sewage treatment effluents. However, much remains to be learned about their ecology before they can be adopted as indicators of faecal pollution and enteric viruses, particularly in groundwater. In individual humans (and in some mammals), FRNA phages are an infrequent component of faeces (Havelaar *et al.* 1986; Furuse 1987; Havelaar 1987; Miller *et al.* 1998). Their abundance in sewage treatment plants may result from the extra-enteral multiplication of the phages at lower temperatures (Havelaar *et al.* 1986; Havelaar 1987; Havelaar and Pot-Hogeboom 1988). Novotny and Lavin (1971) reported that F^+ pili were not synthesized at temperatures lower than 30 °C. Accordingly, because sewage and sewage treatment effluent is generally below this temperature, appreciable phage multiplication should not theoretically be seen. Therefore, the mechanism by which F^+ phages multiply in the sewage environment remains an intriguing, unsolved yet important public health question (Havelaar *et al.* 1990). The extent to which FRNA coliphages can multiply in wastewater facilities is an issue of concern because such multiplication could vary the ratio of indicators to viral pathogens. An important issue that must be resolved is the fact that F^+ phages, which should be specific for *E. coli*, can amplify in environmental coliforms. This non-faecal multiplication limits their use as specific health risk indicators.

Another aspect of sewage in the wastewater environment is resistance of FRNA phages to different sewage treatment processes. While data have been published that show a similarity between FRNA and enteric viral response to chlorination (Havelaar and *et al.* 1985; Havelaar 1986; Sobsey 1989), there have been difficulties in collecting data on the fate of enteroviruses, particularly in treated wastewater. Havelaar *et al.* (1993), showed that the concentration of FRNA coliphages was highly correlated with viral concentrations in most freshwater environments studied. Recently, Vilaginès *et al.* (1996, 1997) studied the occurrence and relationship among enteroviruses, F^+ RNA phages and a variety of bacterial indicators in raw and treated wastewater, and in surface water collected from a receiving river. They found that FRNA phages were less resistant to wastewater treatment than enteric viruses (2·5 $\log_{10}$ elimination rate for phages and 1·4 $\log_{10}$ for viruses). The statistical analysis of the data demonstrated that there was no correlation between the phages recovered and the enteroviruses detected.

A factor motivating the study of bacteriophages as indicators of enteric viruses in groundwater is the hypothesis that the movement characteristics and lifespan of the phages are parallel to enteric viruses. However, little direct evidence has been presented to support this contention. Robertson and Edberg (1997) and Edberg *et al.* (1997) have reviewed the physicochemical characteristics that control the fate and transport of bacteria, viruses and protozoa in groundwater. Key characteristics of the microbe include size, die-off rate and surface electrostatic properties. Key properties of the groundwater/aquifer system include flow velocity, aquifer grain (or pore) size, porosity of the soil, assimilable organic carbon content of the soil and aquifer, other microflora present, water temperature, water pH, and other chemical characteristics of water including its mineral composition. Based on size and surface electrical properties alone, viruses are much more mobile in groundwater than either bacteria or protozoa. The die-off rate of viruses is difficult to measure. However, from laboratory experiments, it appears that viruses are at least as long-lived as vegetative bacteria and maybe more so (Gerba *et al.* 1986, 1991; Harvey and Garabedian 1991; Tim and Mostaghimi 1991; US EPA 1992; Noell 1992; Bales *et al.* 1993). Yates and Yates (1989, 1991) generated numerical models of viral inactivation and found that there was a substantial reduction from 210–325 m away from septic tanks. Maximum migration distances of viruses in groundwater appear to be in the order of 1000–1600 m in channelled limestone and 250–408 m in glacial silt–sand aquifers (Edberg *et al.* 1997). One important factor in ascertaining the lifespan of viruses that has not been sufficiently explored is the differential ability of viruses and other indicators to serve as food for larger prey. It is a law of ecology that the smaller the food source, the more likely it is to be eaten. This law holds for molecules as well as microbes. Therefore, viruses would, theoretically, serve as a better food source and be more readily phagocitized and killed in groundwater than either bacteria or viruses. Certainly, in soil where there is a known, large and diverse microflora, this hypothesis has been substantiated.

Finally, with respect to the origin of the phages themselves,

they are actually indicators of indicators. If the parent indicator (either *E. coli*, a member of the total coliform group, or other Enterobacteriaceae) is not present, the phages will not be present either. Therefore, the correlation between the presence of bacteriophages and enteric viruses on an ecological basis is one step removed from direct correspondence. A few studies have examined actual field data in which coliphages were analysed at the same time as enteric viruses and other indicators. Bakhtar and Yates (1996) detected coliphages on many occasions in the absence of infective enteroviruses (Table 5).

Recently, the US EPA conducted a study that examined faecally-contaminated wells for the presence of enteroviruses, bacteriophages, *E. coli* and *Enterococcus*. It was found that coliphages were no more correlated with the presence of enteroviruses than any other faecal bacterial indicator. Most importantly, when the data were normalized for sample volume (the bacterial indicators were analysed in samples of 100 ml, whereas the coliphages were analysed in samples of 15 l — a 15 000-fold increase in sample volume), bacteriophages were much less likely to correspond. Most importantly, in one of the few studies where actual disease outbreaks (presumed to be caused by enteroviruses) were analysed, the detection of bacteriophages did not correlate well with disease. Interestingly, total coliforms were the best single indicator of viral gastroenteritis obtained from groundwater (Craun *et al.* 1997). It has been argued that this lack of correlation between bacteriophages and either disease outbreak, or the occurrence with enteric viruses, was due to laboratory analytical methods being sub-optimal. However, the investigators chose these methods after a careful review of the literature, and they were widely used at the time. Whether better methods and analytical conditions will yield better correlations between bacteriophages and the presence of enteroviruses and enteroviral disease is an area of intense study, with the results as yet unavailable.

Another major issue of concern in attempting to correlate bacteriophages with enteric viruses is the fact that there are many and varied agents which cause viral gastroenteritis. Some can be grown in culture and some cannot. The most prevalent enteroviruses are the caliciviruses (including the Norwalk agent), rotaviruses, astroviruses and adenoviruses. Hepatitis A and E viruses have also been regularly associated with waterborne disease outbreaks. Until data on the transport and die-off of the variety of enteric viruses are obtained, it will be very difficult to establish whether any of the bacteriophages are adequate indicators. Unfortunately, because of the great difficulty in detecting enteroviruses from human faecal specimens, limited data exist in clinical laboratories concerning the isolation rate of these various agents. In the United States, the Centers for Disease Control and Prevention has established a program in individual locations called the 'Emerging Pathogens Program'. In these areas, resources have been intensely applied to collect public health data. In effect, these geographical sites were chosen as sentinels for the entire country.

3.3 Bacteriophages infecting *Bacteroides fragilis*

Bacteroides fragilis, like *E. coli*, is present in high numbers in the human colon. Bacteriophages active against this anaerobe, particularly *Bacteroides fragilis* strain HSP40, have demonstrated a high degree of host–strain specificity and appear to lack activity against other species of the genus *Bacteroides* (Keller and Traub 1974; Booth *et al.* 1979; Cooper *et al.* 1984; Kory and Booth 1986; Tartera and Jofre 1987; Tartera *et al.* 1989). As a potential indicator of faecal contamination, *B. fragilis* phages were detected extensively in human faeces and sewage and appear to reflect the dominance of the *B. fragilis* host in human faeces (Booth *et al.* 1979; Cooper *et al.* 1984; Salyers 1984; Jofre *et al.* 1986; Tartera and Jofre 1987; Tartera *et al.* 1989; Grabow *et al.* 1995). Phages lytic for the most efficient host strain examined, *B. fragilis* HSP40, were recovered only from environmental areas subjected to sewage and were never detected in non-polluted areas or those occupied exclusively by wild animals (Jofre *et al.* 1986; Tartera and Jofre 1987; Araujo *et al.* 1997).

Gantzer *et al.* (1998b) attempted to determine whether either of two types of bacteriophages (*B. fragilis* phages and somatic coliphages), and/or the detection of the enterovirus genome by genetic amplification, were good indicators of infectious enteroviruses. In the three different types of wastewater tested, *B. fragilis* phages were found to be reliable indicators of enterovirus contamination in defined experimental conditions. These observations, coupled with the apparent inability of *B. fragilis* phages to multiply in fresh water, sea water or sediment habitats (Jofre *et al.* 1986; Tartera *et al.* 1989), suggested that these phages are prominent subjects for exploration as indicators of human faecal pollution and, potentially, enteric virus occurrence.

Phages infecting *B. fragilis* are more resistant to natural inactivation processes than F^+-specific coliphages, which in turn are more resistant than somatic coliphages (Lucena *et al.* 1996; Sun *et al.* 1997). They were also found to be more resistant to water treatment than either somatic or F^+ coliphages and even *Clostridium* spores (Tartera *et al.* 1988; Jofre *et al.* 1995). In a study of more than 1500 drinking water samples from Israel and Spain, the three groups of bacteriophages tested (somatic coliphages, F^+-specific coliphages and *B. fragilis* bacteriophages) were isolated with similar frequencies ranging from 4·4% for somatic coliphages to 6·1% for *B. fragilis* phages (Armon *et al.* 1997). Somatic coliphages, which outnumber the phages infecting *B. fragilis* by more than two orders of magnitude, died off faster than the others. Therefore, it can be concluded that *B. fragilis* phages are much more persistent than somatic coliphages and approximately as resistant as F^+-specific coliphages. However, *B. fragilis* phages are detected in lower concentrations than other phages in fresh water (1–15 pfu $100\,ml^{-1}$) (Araujo *et al.* 1997) and, consequently, it is more

difficult to evaluate the precise ratio of *Bacteroides* phages with enteric viruses. Moreover, there is the need for a concentration step which increases the complexity and time-to-completion of the method. Lastly, the host is an anaerobe, which is more difficult to cultivate than enteric bacteria.

4. SUMMARY

- Low concentrations of all types of bacteriophages in groundwater limit their power to predict the presence of enteric viruses.
- There is little concordance in the literature regarding phage detection methods, thus making comparisons extremely difficult. Different authors have used different hosts, phage concentration methods, and end-point determinations. Also, markedly different volumes of sample have been employed, varying from 1 litre to 400 l.
- Bacteriophage concentration methods are not reproducible. There has been marked variability among groups in the natural substrates used (for example, beef extract), the type of adsorbing filter used, centrifugation instruments and conditions, and the delivery of the concentrate to the host cells.
- There is no consensus on the best bacterial host strain. Currently, several are employed with each showing differential sensitivities and specificities. In particular, host stability must be considered. Host stability has two components: the ability of the host to continue to be receptive to the bacteriophage after continued sub-culture, and the lack of lysogenic or temperate bacteriophage in the host cell line which may be randomly and unpredictably activated.
- There is a lack of consistent recovery of bacteriophages from individual faecal specimens. In particular, only approximately 3% of individual humans carry the FRNA phages. While there is some evidence to indicate that the phages multiply in sewage, it is not clear how they do so since the host pili should not be produced at lower temperatures. These ecological factors need to be understood. Of all the phages thus far studied, *Bacteroides fragilis* HSP40 has the highest recovery rate from individual people. However, *Bacteroides*, being an anaerobe, is a difficult host for routine laboratory analysis.
- Methods for the enumeration of F^+-specific phages and *Bacteroides* phages are complex, time-consuming, costly and not reproducible. Conversely, somatic coliphage methods are simpler and results can be available in 4–6 h.
- The occurrence of phages and viruses in groundwater depends on physicochemical characteristics that control their fate and transport in the groundwater/aquifer environment. There are very little actual data taken from the field that allow an understanding of the ecology and life span of phages in their natural environment. Moreover, the ability of phages to serve as a source of food for other microbes needs to be understood.
- There has been a lack of association of bacteriophage recovery with gastroenteritis outbreaks due to enteric viruses. There is only a small epidemiological database concerning the occurrence of enteric viruses in groundwater.

5. CRITICAL ISSUES THAT MUST BE ADDRESSED

Bacteriophages are appealing as indicators of groundwater enteric virus pollution but their use for this purpose is premature. The following critical issues must be addressed in order for them to meet minimum regulatory requirements.

Methods. The methods are in a state of development. For example, basic issues such as which bacteriophage(s) to choose, the specific bacterial hosts of bacteriophages, analysis conditions, media etc., need to be addressed.

Validation of methods. After choosing the method, it needs to be validated. It is important that commercial and smaller laboratories be included in the protocol. It is particularly important to establish inter- and intra-laboratory reproducibility. A study being conducted by several European laboratories on the validation of ISO methods is scheduled to be released in the year 2000 and should prove useful.

Lack of specificity of bacteriophages. False positives for F^+ coliphages (promoted as being *E. coli* specific) were shown to amplify in non-colonic, environmental coliform bacteria, *Klebsiella pneumoniae* and *Enterobacter cloacae*.

Lack of sensitivity of bacteriophages. F^+ coliphage concentrations reported in septic tank studies were not high. Only 3% of humans have *E. coli* with F^+ coliphages. Coliphages are indicators of indicators. The parent must have been present in the recent past to generate coliphages.

Epidemiological support. There is a lack of association between detection of bacteriophages and disease occurrence.

Cost. The cost of the bacteriophage procedure in a commercial laboratory has not been established.

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