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Potential Usefulness of Bacteriophages That Infect *Bacteroides fragilis* as Model Organisms for Monitoring Virus Removal in Drinking Water Treatment Plants

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The presence of bacteriophages at different stages in three drinking water treatment plants was evaluated to study the usefulness of phages as model organisms for assessing the efficiency of the processes. The bacteriophages tested were somatic coliphages, F-specific coliphages, and phages infecting *Bacteroides fragilis*. The presence of enteroviruses and currently used bacterial indicators was also determined. Most bacteriophages were removed during the prechlorination-flocculation-sedimentation step. In these particular treatment plants, which include prechlorination, phages were, in general, more resistant to the treatment processes than present bacterial indicators, with the exception, in some cases, of clostridia. Bacteriophages infecting *B. fragilis* were found to be more resistant to water treatment than either somatic or F-specific coliphages or even clostridia. Enteric viruses were found only in untreated water in low numbers, and consequently, the efficiency of the plants in the removal of viruses could not be evaluated with precision. The numbers and frequencies of detection of the various microorganisms in water samples taken in the distribution network served by the three plants confirm the results found in the finished water at the plants.

For decades, the fecal coliform group of bacteria has been used as an indicator of water quality regarding the presence of human pathogens. However, the resistance of various pathogenic microorganisms is wide ranging and dependent on many factors, including water treatment. Viruses and some parasites are now recognized as being more resistant to water treatment than the current bacterial indicators of water quality (19, 24, 25). Therefore, it seems necessary to adopt a better indicator with which to monitor the performance of water treatment processes. Potential new indicators include somatic coliphages (15, 16, 22), F-specific bacteriophages (14, 16), and phages infecting *Bacteroides fragilis* (16, 17, 34).

The potential use of coliphages with an indicator function in drinking water treatment processes has already been studied by various authors (3, 12, 20, 23, 30). The present study was undertaken to compare the potential utility of bacteriophages infecting *B. fragilis* with that of somatic and F-specific coliphages as indicators in the monitoring of virus removal in water treatment plants.

MATERIALS AND METHODS

Water treatment plants. Three water treatement plants that produce drinking water for Barcelona and surroundings were included in this study. Particulars regarding the three treatment plants are given in Table 1. Plants A and B draw water with heavy pollution of human origin from the Llobregat river. Plant C draws water with very low levels of pollution from the Ter river, which passes through three water reservoirs and a closed pipeline of 57 km before being drawn for treatment. The performance of these plants regarding the reduction in concentrations of different bacteria is shown in Table 2. Removal of bacteria in the three treatment plants was sufficient to provide a continuous supply of water that fulfills the drinking water quality criteria based on bacterial indicators.

Bacteriophage and virus analysis. *B. fragilis* HSP40, grown on *Bacteroides* phage recovery medium (32) was used in the quantification of *B. fragilis* phages. *Escherichia coli* HS (pF amp R), grown on tryptone agar supplemented with streptomycin (15 mg/liter) and ampicillin (15 mg/liter), was used for the quantification of F-specific coliphages (7). *E. coli* CN13, grown on nutrient agar

TABLE 1. Details of the water treatment plants studied

Plant	Capacity (m^3/day)	Raw water source	Raw water quality[a]	Treatment[b]					
				Prechlorination	Coagulation-sedimentation	Sand filtration	Ozonation	Activated carbon filtration	Post-chlorination
A	300,000	Llobregat river	+++	×[c]	×	×	×	×	×
B	500,000	Llobregat river	+++	×[c]	×	×	−	−	×
C	690,000	Ter river	±	×	×	−	−	×	×

[a] Raw water quality ranged from light (±) to heavy (+++) pollution.
[b] ×, present; −, absent.
[c] Break-point chlorination.

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TABLE 2. Bacteriological data on the three water treatment plants studied

Type of bacteria	Plant A		Plant B		Plant C	
	Bacterial load[a]	Decimal reduction[b]	Bacterial load	Decimal reduction	Bacterial load	Decimal reduction
Total coliforms	5.6	>8.6	5.2	7.1	1.0	2.9
Fecal coliforms	4.9	>7.9	4.8	>7.3	0.8	>3.0
Fecal streptococci	4.2	5.9	4.2	5.1	0.5	2.3
Clostridium spp.	3.1	4.6	3.5	4.2	1.1	>2.7

[a] Logarithm of the mean value of CFU per 100 ml in nontreated water. The mean value was calculated as the arithmetic mean of samples taken weekly over the 2 years in which phages were studied.
[b] Decimal reduction, decrease in logarithms.

containing nalidixic acid (100 mg/liter), was used for the quantification of somatic coliphages (4). *E. coli* strains were kindly provided by R. Armon. All phages were quantified by the double-agar-layer (PFU) method. Coliphages were quantified without any treatment of the sample. Water samples were decontaminated by filtration through polyvinylidene difluoride membrane filters (Millipore) as described elsewhere (32) before quantification of phages infecting *B. fragilis*. Quantifications were performed with 10 ml of sample.

Additionally, presence-absence tests in 100 ml of sample were performed as follows. A screw-capped bottle of 250 ml containing 120 ml of double-concentrated medium was inoculated with 100 ml of the sample and 30 ml of a culture of the host bacterium. The mixture was then incubated at 37°C for 24 h. Following incubation, 0.7 ml of the culture was mixed with 0.3 ml of chloroform, shaken vigorously for 2 min, and centrifuged at 16,000 × *g* for 1 min. Finally, the presence of bacteriophages in the supernatant was determined by the spot test. For phages infecting *B. fragilis*, the 100 ml of sample was treated to reduce, as far as possible, the oxygen present in the water by bubbling nitrogen for 15 min at a flow rate of 5 liters/min.

Enteroviruses were quantified by plaque formation by inoculating confluent BGM cell monolayers as described elsewhere (5).

Concentration of virus. Viruses from 50 liters of river water were concentrated by the glass powder adsorption-elution method (27). To evaluate the presence of viruses in finished water and water in the distribution network, these were concentrated from 1,000 liters with the electropositive filter (Z-plus 50S; Cuno) adsorption-elution method (29). The concentrate was reconcentrated by organic flocculation (18).

Bacteriological analysis. Total coliforms, fecal coliforms, and fecal streptococci were enumerated by the Standard Methods most probable number procedures (2) in raw water from plants A and B and by Standard Methods membrane filter procedures (2) for all of the other samples. Sulfite-reducing clostridia were enumerated by a procedure described by Button (6).

Statistical analysis. An analysis of variance test was applied to raw water data. For the results of treated water, which are dichotomous, i.e., only positive or negative, nonparametric tests were performed. The Friedman test, Cochran Q test, and Kendall's concordance coefficient were used. In those cases in which the tests mentioned above indicated significant differences between the different parameters, the Wilcoxon test was applied to discern which groups of phages were significantly different from the others. All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, Ill.).

RESULTS

Bacteriophage and virus levels in raw water. Levels of bacteriophages and enteroviruses in raw water at the three treatment plants are shown in Table 3. In plants A and B, the three bacteriophages studied were present in sufficient quantities to be detected in all of the 10-ml samples by the double-agar-layer techniques, whereas in plant C they were not always detected in 10-ml samples. In untreated water entering plants A and B, somatic coliphages and F-specific coliphages significantly ($P < 0.001$, by analysis of variance) outnumbered phages infecting *B. fragilis*, whereas raw water samples from plant C contained numbers of the three types of phages which were of the same order of magnitude and not significantly ($P < 0.05$, by analysis of variance) different. Human enteric viruses were detected in untreated waters in plants A and B but never in plant C. The numbers of enteroviruses detected in raw water entering plants A and B ranged from <20 to 630/1,000 liters (plant A) and 158/1,000 liters (plant B).

Removal of bacteriophages and enteric viruses. Bacteriophages were never detected in 10 ml of any water sample taken from any plant after the prechlorination-flocculation-sedimentation step when analyzed by the double-agar-layer method. Enteric viruses were also never detected in concentrates of 1,000 liters of any water sample taken after the first treatment step (prechlorination-flocculation-sedimentation) in any of the three plants.

However, the different kinds of bacteriophage were detected by presence-absence tests in variable percentages of 100-ml samples of water taken after the different stages of water treatment in all three plants, including finished waters (Fig. 1 to 3). Similar trends were observed in all three plants. First, most phages were removed in the first step of treatment. Second, phages were isolated on more (plants A and B) or the same number (plant C) of occasions after step 2 than or as after step 1. We do not have a proven explanation for this unexpected observation; however, a breakup of aggregates not sedimented in step 1 may explain it. Third, at the end of the three treatment processes, phages infecting *B. fragilis* were isolated on more occasions than somatic and F-specific coliphages, although the differences were not significant (tests for nonparametric data, $P < 0.05$) when data from each plant were treated

TABLE 3. Levels of phages and enteroviruses in raw water of plants A, B, and C

Parameter	Plant A (n = 24)			Plant B (n = 24)			Plant C (n = 20)		
	Log_{10} PFU[a]		% Positive samples	Log_{10} PFU[a]		% Positive samples	Log_{10} PFU[a]		% Positive samples
	Mean	Max–min[b]		Mean	Max–min		Mean	Max–min	
Somatic coliphages	4.3	5.6–3.1	100	3.9	4.5–1.5	100	1.3	2.6–BDL[c]	35
F-specific coliphages	3.8	5.5–1.3	100	3.5	4.4–1.6	100	1.1	2.6–BDL	31.8
Phages of *B. fragilis*	2.1	3.1–1.0	100	2.1	3.2–1.6	100	1.0	2.0–BDL	36.4
Enteric viruses	2.1	2.8–BDL	55	1.5	2.2–BDL	50	BDL	BDL–BDL	0

[a] For somatic coliphages, F-specific coliphages, and phages of *B. fragilis*, the values are per 100 ml; for enteric viruses, the values are per 1,000 liters.
[b] Range from maximum to minimum.
[c] BDL, below detection limit.

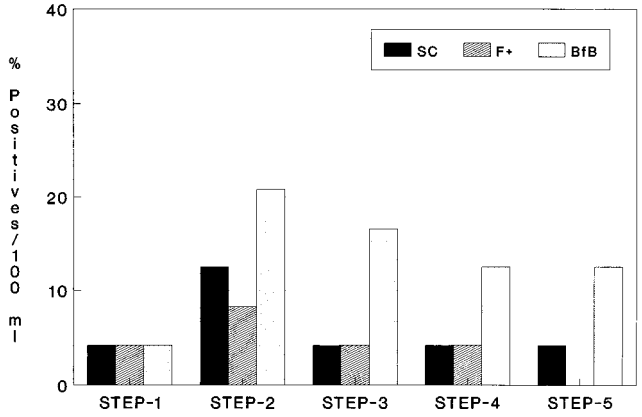

FIG. 1. Percentage of positive isolations of bacteriophages in 100-ml water samples during and after treatment in plant A. Steps: 1, water after prechlorination-flocculation-sedimentation; 2, water after sand filtration; 3, water after ozonation; 4, water after granular activated carbon filtration; 5, water after postchlorination. Abbreviations: SC, somatic coliphages; F+, F-specific coliphages; BfB, *B. fragilis* bacteriophages. Numbers of samples, 24.

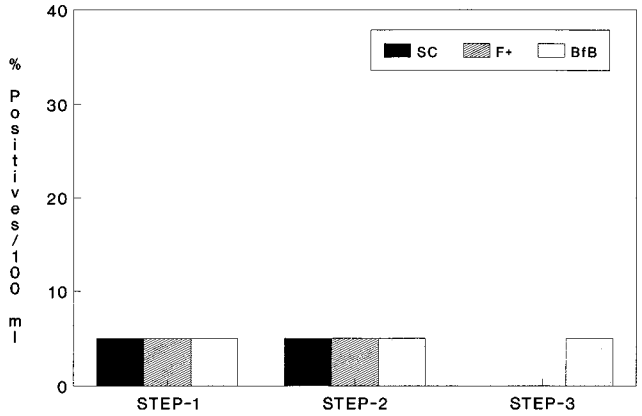

FIG. 3. Percentage of positive isolations of bacteriophages in 100-ml water samples during and after treatment in plant C. Steps: 1, water after prechlorination-flocculation-sedimentation; 2, water after granular activated carbon filtration; 3, water after postchlorination. Abbreviations: SC, somatic coliphages; F+, F-specific coliphages; BfB, *B. fragilis* bacteriophages. Numbers of samples, 20.

independently. However, when the 68 samples of finished water from the three treatment plants are considered together, the percentages of positive isolations were 11.7% for phages infecting *B. fragilis*, 2.8% for somatic coliphages, and 1.4% for F-specific coliphages. In this case, the differences among the three groups of phages were significant ($P < 0.05$) for the Cochran Q test and the Kendall's concordance coefficient, whereas only the difference between *B. fragilis* phages and F-specific coliphages was significant by the Wilcoxon matched-pair signed rank test.

Since the differences between the amounts of the different groups of phages were significant in raw water and nonsignificant in finished water, the difference in inactivation should be considered significant. In plant C, where levels of prechlorination were lower than those in plants A and B, the difference in the removal of phages infecting *B. fragilis* and F-specific phages is not significant.

Decimal reduction of some of the groups of bacteriophages could not be calculated in some plants because they were not detected at the end of the process, and consequently, the only data available show that their removal was greater than certain given values. However, some trends can be observed. Decimal reduction of phages after the full treatment decreased from plant A to plants B and C in accordance with the extent of the water treatment (Table 4). In treatment plants A and B, removal of phages infecting *B. fragilis* was much less efficient than removal of either somatic coliphages or F-specific coliphages in all plants (Table 4).

The efficiency of enterovirus removal could not be calculated accurately because of the low levels of enteric viruses in the raw waters and their consequent absence in treated water samples.

Microbial levels in distribution water. Tests were also performed on 118 drinking water samples taken at different points of the distribution network served by the three water treatment plants (Table 5). These samples showed lower percentages of positive presences of the different phages in 100 ml than the samples of finished water did. In the set of samples analyzed, phages infecting *B. fragilis* were isolated on more occasions than either somatic coliphages or F-specific coliphages, although these differences were not significant ($P < 0.05$) by the test applied for nonparametric data. Interestingly, phages infecting *B. fragilis* were isolated even more frequently than sulfite-reducing clostridia. Neither fecal coliforms nor enteroviruses were detected in this set of samples.

When data from finished water from the plants and data from the distribution network were analyzed statistically as a

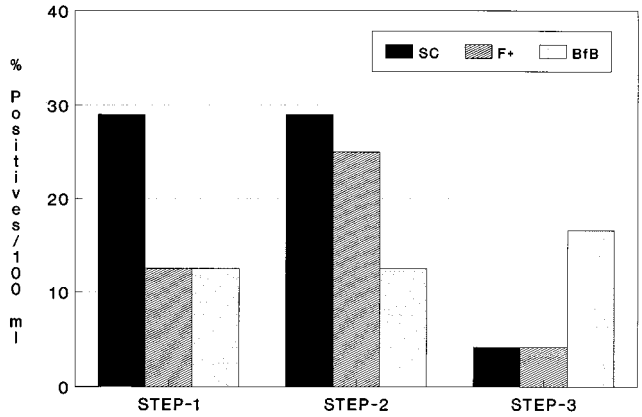

FIG. 2. Percentage of positive isolations of bacteriophages in 100-ml water samples during and after treatment in plant B. Steps: 1, water after prechlorination-flocculation-sedimentation; 2, water after sand filtration; 3, water after postchlorination. Abbreviations: SC, somatic coliphages; F+, F-specific coliphages; BfB, *B. fragilis* bacteriophages. Numbers of samples, 24.

TABLE 4. Decimal reduction of phages and enteroviruses and after the complete treatment in the three water treatment plants

Virus category	Decimal reduction[a]		
	Plant A	Plant B	Plant C
Somatic coliphages	5.6	>5.3	>2.6
F-specific coliphages	5.2	4.9	2.3
Phages of *B. fragilis*	2.9	2.7	2.2
Enteric viruses	>3.4	>2.9	

[a] Decimal reduction, decrease in logarithms. Numbers indicate the decimal reduction calculating the value of phages present in finished water by Thomas' formula for the calculation of the most probable number for long series of data (8).

TABLE 5. Percentage of distribution network samples from which bacterial indicators and phages were isolated in 100 ml of water

Parameter	No. of samples	% of samples positive	CFU or PFU
Fecal coliforms	118	0	0
Fecal streptococci	118	2.5	0.025[a]
Clostridia	118	1.7	0.016[a]
Somatic coliphages	118	1.7	0.017[b]
F-specific coliphages	118	2.5	0.025[b]
Phages of *B. fragilis*	118	4.2	0.043[b]
Enteric viruses	118	0	0[c]

[a] Per 100 ml.
[b] Per 100 ml. Values calculated by Thomas' formula for the calculation of the most probable number for long series of data.
[c] Per 1,000 liters.

single group of data, then the differences between phages infecting *B. fragilis* and both somatic coliphages and F-specific coliphages were significant ($P < 0.05$), whereas the difference between somatic coliphages and F-specific coliphages was not significant by the Wilcoxon test.

DISCUSSION

In spite of the heterogeneity of the plants regarding the treatment processes, there are several observations about removal of bacteriophages that apply to all three and which are valid for bacteriophages.

First, regarding the comparative removal of current bacterial indicators and bacteriophages, data show that fecal coliforms and fecal streptococci were removed more efficiently than the three types of phages, as already described for coliphages by other authors (20, 35). In contrast, available information indicates that clostridia were more resistant to treatment than coliphages (23). However, the data presented here show that at least in plants A and B, clostridia were removed more efficiently than phages infecting *B. fragilis*, probably as a consequence of the filtration steps.

Second, in all treatment plants, prechlorination-flocculation-sedimentation was the most efficient step in reducing the numbers of phages. This step has been described by other authors as highly efficient for the removal of viruses and bacteriophages (24). This was more evident in plants A and B, where treatments include break point chlorination, which has been described as very effective in coliphage reduction (11). Bacteriophages, mainly those infecting *B. fragilis*, that survive the first step seem to pass easily through all of the subsequent steps in the process. Indeed, elimination of some particular coliphages by sand filtration and adsorption to activated carbon has been described previously to be low and inconsistent, as has also been described for human enteric viruses (10, 12). There is no obvious explanation for this, although it may be related to disaggregation phenomena. The low efficiency of phage removal by ozonation may seem surprising, since in laboratory experiments ozonation was shown to be very efficient in virus and phage elimination (19, 21). However, the dose of ozone used in this plant is applied for the oxidation of organic chemicals rather than for disinfection. Moreover, some available data for large plants show results for enteric viruses similar to those reported here, in that no significant differences can be observed in water pre- and postozonation (25). Third, it is also evident that the three kinds of phages studied were removed with different efficiencies, at least in plants A and B. In the treatment processes studied, which include prechlorination, phages infecting *B. fragilis* were more resistant to treatment than either somatic coliphages or F-specific bacteriophages and F-specific coliphages were more resistant than somatic coliphages. Considering that the most important factor in phage removal is chlorination, our results agree with previous data indicating that F-specific coliphages are more resistant to chlorination than somatic coliphages (13) and that phages infecting *B. fragilis* are more resistant to chlorine disinfection than certain F-specific bacteriophages (33).

Data from the distribution network confirm the data from the tests performed on finished water and coincide with existing data which show that somatic coliphages are more abundant than fecal bacteria in drinking water samples from distribution networks (9, 26).

Human enteric viruses were removed to such an extent than we could not detect them after the first step in any of the plants in which they were present in the untreated water, thus confirming that levels of human enteric viruses in raw waters are too low to be used to monitor virus removal by different water treatments, as pointed out by other authors (23). Available data indicate that human enteric viruses are more resistant to water treatment than current bacterial indicators (20, 25, 31). Moreover, some human enteric viruses, such as rotaviruses, have been reported to survive better than somatic coliphages and other enteric viruses such as enteroviruses (20). Data presented here show that, in these particular drinking water treatment plants, which include prechlorination, phages infecting *B. fragilis* are more resistant to water treatment than enteroviruses and consequently may behave similarly to other enteric viruses such as rotaviruses and hepatitis A viruses. On the basis of data obtained in disinfection experiments (1, 28), rotaviruses and hepatitis A viruses seem to be more resistant than enteroviruses to chlorination. Moreover, it has also been shown in disinfection experiments that resistance to chlorination of phage B40-8 infecting *B. fragilis* is intermediate between values of poliovirus and those of rotaviruses and hepatitis A virus (1, 33).

In conclusion, phages infecting *B. fragilis* are more resistant to some water treatments than are coliphages, they are significantly more abundant than enteroviruses in pretreated waters, and although they are not as abundant as coliphages in pretreated waters, their numbers are high enough to allow their isolation in treated waters. Consequently, phages infecting *B. fragilis* deserve further attention as model organisms for the evaluation of removal of human enteric viruses in water treatment plants.

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