Application of Direct Plaque Assay for Detection and Enumeration of Bacteriophages of *Bacteroides fragilis* from Contaminated-Water Samples

ROBERTO CORNAX, MIGUEL A. MORIÑIGO, IGNACIO G. PAEZ, MARÍA A. MUÑOZ,
AND JUAN J. BORREGO*

*Department of Microbiology, Faculty of Sciences, University of Malaga,
Campus Universitario Teatinos, 29071-Málaga, Spain*

The direct double-agar-layer plaque assay for the detection and enumeration of specific bacteriophages of *Bacteroides fragilis* from contaminated-water samples was performed. Several factors that affect the methods, such as conditions of the bacterial culture, composition of the assay medium, addition of divalent cations, and decontamination techniques applied to the sample, were evaluated. The results obtained show that the direct assay technique proved to be more efficient than the most-probable-number technique. A higher recovery of bacteriophages was obtained from 17 of 24 samples with the direct assay. The two methods only showed similar results from samples with a low degree of pollution.

Bacteriophages of *Escherichia coli* are considered reliable indicators of the fecal pollution of natural waters (3, 4), although their use as viral indicators could also be very interesting. The proposal that these bacteriophages may be useful as indicators of enteric viruses is based on several facts: they are detected in high numbers in sewage and polluted waters, they have similar resistances to enteric viruses in aquatic environments and in water treatment processes, and their detection is carried out by simple, inexpensive and rapid techniques (7, 10, 18). However, Vaughn and Metcalf (17) suggested that the use of coliphages as indicators was not appropriate because of their low correlation with the presence of human enteric viruses in water and because of the possible multiplication of the coliphages in natural waters in certain seasons.

In view of these contradictory opinions, Tartera and Jofre (15) have proposed the *Bacteroides fragilis*-specific bacteriophages as ideal viral indicators. These bacteriophages are detected in only 10% of the fecal samples, those exclusively from human origin; they are also detected in sewage and contaminated natural waters but never in non-fecally contaminated samples. Moreover, *B. fragilis* is a strict anaerobe, and thus, phage multiplication in the aquatic environment is unlikely (16).

Since the proposal of the use of coliphages as viral indicators (10), several investigators have developed methods to detect and enumerate coliphages in water. The method widely used for the enumeration of phages is based upon the double-agar-layer technique described by Adams (1). Tartera and Jofre (15) found low efficiency in the enumeration of *B. fragilis* phages from natural waters by using this technique, and they proposed a modification of the multiple-tube technique described by Kott (9) as alternative method.

In this paper, we evaluate several factors that affect the detection and enumeration of *B. fragilis* phages by the direct assay of the double-agar-layer technique.

MATERIALS AND METHODS

Sampling. Sewage samples were taken from an outfall that discharged directly at the Huelin beach (Málaga, Spain). Seawater samples with different degrees of fecal contamination were obtained from the same beach 10, 100 (high degree of fecal pollution), 500, and 1,000 m (moderate or low degree of fecal pollution) from the sewage discharge point. The geometric means (in CFU per 100 ml) of fecal coliform counts in these sampling points were as follows: 10 m, 1.9×10^6; 100 m, 1.0×10^4; 500 m, 2.8×10^3; and 1,000 m, 2.2×10^2. A total of 30 samples with different degrees of fecal contamination were tested. All the samples were collected in sterile glass bottles, transported to the laboratory under refrigeration, and analyzed within 4 h of the sampling.

Bacterial strains and culture conditions. *B. fragilis* HSP40 (15) was used as specific host bacterial strain in all phage assays. The bacterial host was grown in modified brucella broth (MBB), the composition of which (in grams per liter) is as follows: brucella broth (Difco Laboratories, Detroit, Mich.), 28; L-cystine (E. Merck AG, Darmstadt, Federal Republic of Germany), 0.005; magnesium sulfate, 7; H_2O (Sigma Chemical Co., St. Louis, Mo.), 0.12; and calcium chloride (Sigma), 0.05.

The following bacterial culture conditions were compared: (i) culture in 100-ml vials of MBB sealed with a rubber plug and metal cap; (ii) culture in vials of MBB with an anaerobic atmosphere, achieved by bubbling CO_2 (20%) and N_2 (80%) through the culture; and (iii) culture under anaerobic conditions using GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) with an H_2-CO_2 atmosphere (BioMerieux S.A., Madrid, Spain).

The growth curves were determined by inoculation of a 48-h culture of *B. fragilis* by using a sterile hypodermic syringe in the different vials. The titration of the culture was performed at 6-h intervals by counting the CFU in modified blood agar base (MBAB) plates, by using the dissemination technique with a sterilized rod. The counts were obtained after 48 h of incubation under anaerobic conditions.

Media. The following media were evaluated for the enumeration of *B. fragilis* phages by using the double-agar-layer technique: (i) MBAB (15), which contains 40 g of blood agar

* Corresponding author.

base no. 2 (Difco) and 0.05 g of L-cystine (Merck) per liter; (ii) MBAB-sorbate (MBAB-S), similar in composition to MBAB but supplemented with 0.05% potassium sorbate (Sigma) and adjusted to pH 5.7 with 0.01 N hypochloric acid (Sigma); (iii) MBAB medium supplemented with 500 U of sodium penicillin (Sigma) per liter and 500 µg of gentamicin sulfate (Sigma) per ml (MBAB-PG); (iv) MBAB medium supplemented with 100 µg of kanamycin sulfate (Sigma) and 7.5 µg of vancomycin (Sigma) per ml (MBAB-KV); and (v) modified Schaedler agar (MSA-KV), which contains (per liter of distilled water) 40 g of Schaedler broth (BioMerieux), 14 g of agar-agar (Difco), 1 g of esculin (Difco), 0.5 g of ferrium citrate (Sigma), 100 mg of kanamycin sulfate (Sigma), and 7.5 mg of vancomycin (Sigma). The soft agar used in the double-agar-layer technique was prepared by adding 5 g of agar-agar (Difco) to 1 liter of MBB.

A study testing different concentrations of calcium and magnesium ions was carried out to determine the optimal concentrations of both ions for the phage assay. All the media tested were then supplemented with 0.5 mM concentrations of these ions. The results obtained were expressed as relative recovery efficiency percentages, calculated by dividing the phage recovery obtained with a given ion concentration by the maximal phage recovery obtained and multiplying the result by 100.

Phage assay. The double-agar-layer plaque assay was carried out as follows. A 1-ml portion of the water sample or dilutions and 1 ml of the bacterial host culture (10^7 CFU/ml) were added to 3 ml of soft agar melted and maintained at 45°C. The mix was poured onto agar plates and, immediately after solidifying, the plates were put into an anaerobic jar with an H_2-CO_2 atmosphere and incubated at 36°C for 18 to 24 h.

To eliminate the background flora of the samples, several methods were used, such as chloroform treatment (20%) (vol/vol), filtration through membrane filters (47-mm diameter, 0.45-µm pore size) (Millipore Corp., Bedford, Mass.), and filtration through membrane filters of 0.45- or 0.22-µm pore size (Millipore HA 47 mm) previously treated with 10 ml of 3% beef extract (Difco) at pH 9.5.

The efficiency of the direct plaque assay was compared with that of the multiple-tube technique proposed by Tartera and Jofre (15). Three series of three screw-cap tubes (30 ml) containing 10 ml of MBB (2.5-fold concentrated), 19 ml of single-strength MBB, and 20 ml of single-strength MBB were inoculated with 10, 1, and 0.1 ml of the sample, respectively. When heavily polluted samples were examined, 1 ml of adequate dilutions was also assayed. All the tubes were inoculated with 10 ml of the bacterial culture and incubated at 36°C for 36 to 48 h. After this period, 5-ml samples from each tube were centrifuged at 2,000 × *g*. The pellets were discarded, and the supernatants were treated with chloroform. A drop of each treated supernatant was dispensed on plates of MBAB-S. The plates were incubated in an anaerobic atmosphere at 36°C for 18 h. The titration of the number of phages present in the sample was carried out by using most-probable-number tables (2).

Statistical analysis. Because the counts obtained did not follow a normal distribution, two nonparametric tests (14) were chosen. When only two treatments were compared, the Wilcoxon range test was used (StatView 512 Plus program for Apple Macintosh), and for more than two treatments, the concordance coefficient of the Kendall test was selected.
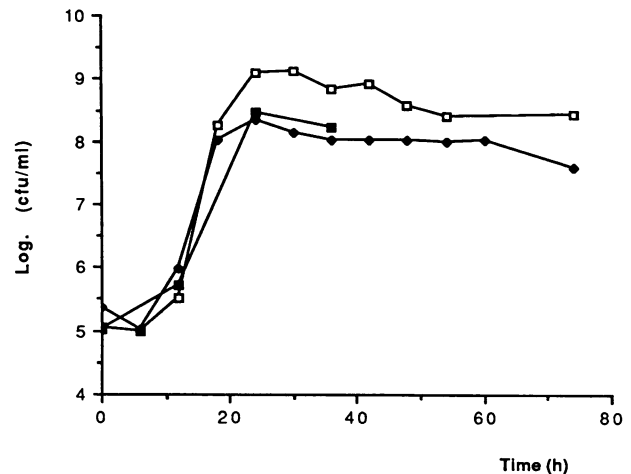

FIG. 1. Growth curves of *B. fragilis* HSP40 in different culture conditions. Symbols: ◆, culture in vials sealed with rubber plug and metal cap; □, culture in vials with an anaerobic atmosphere achieved by bubbling of CO_2 (20%) and N_2 (80%) through the culture; ■, culture under anaerobic conditions using GasPak jars.

RESULTS

Figure 1 represents the growth curves of *B. fragilis* HSP40 in the different culture conditions tested. Over the first 12 h, there was a lag period in which the microbial density did not increase. After this period, a log period was observed, with the fastest growth rate occurring in the vials with a CO_2-N_2 atmosphere. Under these conditions, the highest microbial titer (1.38×10^9 CFU/ml) was also obtained. When vials cultured without gas bubbling were used, a decrease of more than 1 order of magnitude in the final bacterial titer was observed.

The influence of the addition of selected concentrations of calcium and magnesium ions on the *Bacteroides* phage assay in shown in Fig. 2. The addition of both ions increased the efficiency of the bacteriophage recovery, and the highest relative recovery efficiency was obtained at concentrations of 0.5 mM calcium and 0.5 mM magnesium, although no clear difference was seen if only the calcium ion (at the same concentration) was added.
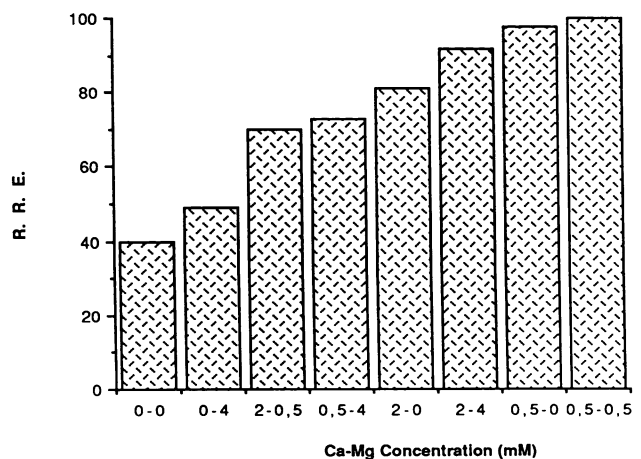

FIG. 2. Relative recovery efficiency as a function of the concentrations of calcium and magnesium cations.

TABLE 1. Concordance coefficient of Kendall analysis applied to the decontamination techniques used

Technique	Bacteriophage count (10^3)/100 ml[a] in:							
	Sewage				Polluted seawater			
	Range	Geometric mean	BF	Rj	Range	Geometric mean	BF	Rj
Chloroform	17–24	20	++	10	7.2–12	10	++	10
Membrane filtration	3.8–6.9	4.8	+	15	1.6–3.6	2.6	−	15
Filters plus beef extract	82–93	89	+	5	5.9–77	72	−	5
Kendall coefficient (W)				1[b]				1[b]

[a] Results from 5 experiments, done in triplicate. BF, Background flora degree; Rj, Kendall cumulative ranges; − to +++, lesser to higher degree.
[b] Significant at level $P < 0.01$

The results obtained from the decontamination techniques are given in Table 1. Filtration through nitrocellulose filters previously treated with 3% beef extract at pH 9.5 proved to be the most effective method of suppressing the background flora, and with this method the highest recovery rate of bacteriophages was also obtained (more than one order of magnitude). The use of 0.45-μm-pore-size filters showed a higher efficiency of phage recovery than the use of 0.22-μm-pore-size filters (Table 2).

The concordance coefficient of Kendall was applied to compare the culture media tested. All the samples were previously decontaminated by membrane filtration (membrane filters of 0.45 μm, treated with beef extract). For sewage samples, the Kendall coefficients obtained among the different culture media were not significant. A higher phage concentration from polluted seawater was recovered by using MBAB, although if all the samples are considered, there is no significant difference among the several media used.

From the 30 samples with different degrees of fecal pollution tested for the detection and enumeration of B. fragilis phages by using both the direct assay technique and the multiple-tube method, it was possible to isolate specific bacteriophages in 24 by one or both methods. The direct assay using the double-agar-layer technique was the best method in almost all the samples, except in those collected from a source of low fecal pollution, in which significant differences between both methods were not observed (Table 3).

DISCUSSION

B. fragilis HSP40 is a strictly anaerobic microorganism, so the optimal technique for its culture requires the use of a gas atmosphere. In the present study, it was demonstrated that the culture of this microorganism in vials with a CO_2-N_2 atmosphere allows the optimal growth, compared with culture in anaerobic jars or in vials without gas bubbling prior to the culture.

TABLE 2. Recovery of B. fragilis HSP40 bacteriophages from sewage by using the membrane filtration technique[a]

Pore size of filters (μm)[b]	Geometric mean of counts (10^3)	Range of counts (10^3)	No. of highest count[c]
0.22	15	7.0–23	0
0.45	24	19–27	6

[a] Each filter was used in 6 experiments, done in triplicate. The Wilcoxon coefficient (Z) was −2.207 ($P < 0.05$).
[b] Filters were previously treated with 3% beef extract (pH 9.5).
[c] Number of tests in which a higher count was obtained than by the other method.

The media used for the direct enumeration of specific bacteriophages of B. fragilis were very complex and rich in nutrients. It is therefore common for background flora to grow in these media, which hampers the optimal lysis plaque visualization. To eliminate the contaminating background flora, chemical treatment with chloroform was used, which produces a low rate of decontamination of the sample and a lower recovery of Bacteroides phages, although Tartera and Jofre (15) indicated a high resistance of these phages to chloroform. However, other authors have reported a decrease of the phage recovery after treatment of the samples with this chemical (8).

Use of membrane filters is very efficient in decreasing the background flora, but it also negatively affects the phage titer. The mechanisms that are involved are still unknown, but the phages may be retained on the filters by means of electrostatic and hydrophobic interactions (5, 6, 13). To avoid these interactions between filters and viruses, the membrane filters were treated with 3% beef extract at pH 9.5. The proteins of the beef extract possess a higher diffusion ability than the phages, so an affinity for the adsorption of virus to pore walls is established (11). In the same way, the increase of pH to values greater than the virus isoelectrical point provokes repulsion forces with the filters.

TABLE 3. Comparative recovery of B. fragilis bacteriophages from natural waters using most-probable-number and direct assay techniques

Source of samples and method[a]	No. of expts	Geometric mean of counts (10^2)	Range of counts (10^2)	No. of highest count[b]	Wilcoxon coefficient (Z)[c]
Sewage					
D	7	631	10–2,600	5	−1.99*
MPN		240	1–1,100	1	
Highly polluted seawater					
D	8	320	1–1,000	7	−1.96*
MPN		90	1–240	1	
Moderately or low-polluted seawater					
D	9	23	0.1–200	5	−1.4
MPN		0.82	0.07–24	3	

[a] D, Direct assay by the double-agar-layer method; MPN, most probable number by the multiple-tube method. For the direct assay, the samples were decontaminated by using filters treated with beef extract.
[b] Number of tests in which a higher count was obtained than by the other method.
[c] The Wilcoxon coefficient for the total of all experiments was −2.77 at significance level $P < 0.05$. *, $P < 0.05$.

By using this technique, a significant increase in the bacteriophage count was obtained from the samples. Filters of 0.22-μm pore size, used by Tartera and Jofre (15) to decontaminate the samples, showed a tendency to block solid matter contained in natural water samples; for this reason, 0.45-μm-pore-size filters proved to be more efficient.

It is very important to determine the optimal ionic concentration of the culture medium for the bacteriophage adsorption and penetration processes (1). Although from the results obtained in this study it may seem that only the addition of calcium ions to the medium is sufficient to produce a good bacteriophage-host interaction, the possible presence of magnesium-dependent phages (12) makes the use of a calcium-magnesium combination at a 0.5 mM concentration of each ion advisable. Similar concentrations of both ions were proposed by Tartera and Jofre (15).

Previously, it was noted that the background flora was reduced considerably by using membrane filtration with beef extract treatment. However, with heavily contaminated samples, the combined use of any decontamination method and of assay in a selective medium is suggested. With regards to the recovery efficiency, no significant differences were observed among the culture media used with sewage samples, although the addition of kanamycin and vancomycin increased the phage recovery rate. For samples with a lower degree of pollution, MBAB is the chosen medium.

In short, on the basis of the results obtained, we propose the following technique of direct assay of *B. fragilis* bacteriophages. Cells of the host bacteria, *B. fragilis*, should be cultured in MBB vials with an atmosphere of CO_2 (20%) and N_2 (80%). Sample decontamination should be performed by using membrane filtration (0.45-μm pore size) with membranes previously treated with 10 ml of 3% beef extract (pH 9.5). MBAB should be used as the bottom agar layer. In the case of samples with a high degree of pollution, supplementing the medium with kanamycin and vancomycin (MBAB-KV) is advised.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. American Public Health Association. 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
3. Borrego, J. J., R. Cornax, M. A. Moriñigo, E. Martínez-Manzanares, and P. Romero. 1989. Coliphages as an indicator of faecal pollution in water. Their survival and productive infectivity in natural aquatic environments. Water Res. 24:111–116.
4. Borrego, J. J., M. A. Moriñigo, A. de Vicente, R. Cornax, and P. Romero. 1987. Coliphages as an indicator of faecal pollution in water, its relationship with indicator and pathogenic microorganisms. Water Res. 21:1473–1480.
5. Farrah, S. R. 1982. Chemical factors influencing adsorption of bacteriophage MS2 to membrane filters. Appl. Environ. Microbiol. 43:659–663.
6. Farrah, S. R., and G. Bitton. 1978. Elution of poliovirus adsorbed to membrane filters. Appl. Environ. Microbiol. 36:982–984.
7. Grabow, W. O. K., P. Coubrough, E. M. Nupen, and B. W. Bareman. 1984. Evaluation of coliphages as indicators of the virological quality of sewage-polluted water. Water S. A. 10:7–14.
8. Kennedy, J. E., Jr., G. Bitton, and J. L. Oblinger. 1985. Comparison of selective media for assay of coliphages in sewage effluent and lake water. Appl. Environ. Microbiol. 49:33–36.
9. Kott, Y. 1966. Estimation of low numbers of *Escherichia coli* bacteriophage by use of the most probable number method. Appl. Microbiol. 14:141–144.
10. Kott, Y., N. Roze, S. Sperber, and N. Betzer. 1974. Bacteriophages as viral pollution indicators. Water Res. 8:165–171.
11. Mix, T. W. 1987. Mechanism of adsorption and elution of viruses to and from surfaces, p. 127–139. *In* G. Berg (ed.), Methods for recovering viruses from the environment. CRC Press, Inc., Boca Raton, Fla.
12. Puck, T. T., A. Garen, and J. Cline. 1951. Mechanism of virus attachment to host cells: role of ions in a primary reaction. J. Exp. Med. 93:65–88.
13. Shields, P. A., and S. R. Farrah. 1983. Influence of salts on electrostatic interactions between poliovirus and membrane filters. Appl. Environ. Microbiol. 45:526–531.
14. Siegel, S. 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Co., New York.
15. Tartera, C., and J. Jofre. 1987. Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. Appl. Environ. Microbiol. 53:1632–1637.
16. Tartera, C., F. Lucena, and J. Jofre. 1989. Human origin of *Bacteroides fragilis* bacteriophages present in the environment. Appl. Environ. Microbiol. 55:2696–2701.
17. Vaughn, J. M., and T. G. Metcalf. 1974. Coliphages as indicators of enteric viruses in shellfish and shellfish raising estuarine waters. Water Res. 8:613–616.
18. Yates, M. V., C. P. Gerba, and L. M. Kelley. 1985. Virus persistence in groundwater. Appl. Environ. Microbiol. 49:778–781.