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Culture and Decontamination Methods Affecting Enumeration of Phages Infecting *Bacteroides fragilis* in Sewage

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A new medium has been adapted for the growth of *Bacteroides fragilis* so that its phages can be recovered from environmental samples, and its efficiency has been assessed. Polyvinylidene difluoride membranes allow significantly higher recoveries among different membrane filters used to decontaminate the samples. In all cases, a number of phages remain in the filters and a percentage of them can be recovered by treatment with an eluant.

Water has been documented as a vector of viral diseases, but many of the agents primarily involved are difficult to detect in water (3, 9, 16, 20, 24, 26, 35). For practical purposes such as monitoring of established treatment processes or the formulation of quality standards for water and shellfish, a model organism would be desirable. Three groups of phages are currently investigated in this respect, namely, the somatic and the RNA F-specific coliphages (11, 12, 14, 15, 27, 28, 30) and the phages of *Bacteroides fragilis* (17, 32). According to data available, phages of *B. fragilis* could be good surrogate indicators for human viruses (16, 17, 31, 33) and they may also be used to distinguish between human and animal fecal pollution (33). To facilitate further investigations, we aimed to improve culture conditions of *B. fragilis* so that bacteriophages could be recovered from environmental water samples.

Cultural conditions have been determined which make *B. fragilis* easy to use for enumerating its phages. Moreover, assay conditions have been established to improve phage recovery while decontaminating the sample by filtration.

B. fragilis HSP40 and MBB and MBAB media were described previously (32). The medium described herein, *Bacteroides* phage recovery medium (BPRM), contained the following per liter: pancreatic digest of casein, 10 g; peptone from meat, 10 g; yeast extract, 2 g; NaCl, 5 g; $CaCl_2$, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; L-cysteine hydrochloride monohydrate, 0.5 g; glucose, 1.8 g; hemin, 10 ml of a 0.1% (wt/vol) solution made up in NaOH 0.02%; 1 M Na_2CO_3, 25 ml; and water, 965 ml. Hemin and Na_2CO_3 solutions were filtered and added to the medium after sterilization at 121°C for 15 min. The pH was adjusted to 7 with concentrated HCl. Kanamycin (100 mg/liter) and vancomycin (7.5 mg/liter) were added to BPRM to prevent the growth of unwanted background bacteria (8, 21). Anaerobic conditions were obtained as described previously (32). The temperature of incubation was 37°C.

Phage B40-8 was selected as the model organism since its morphology is most typical of phages infecting *B. fragilis* HSP40 isolates in environmental samples. B40-8 particles have an isometric icosahedral head (51 nm) and a long (113-nm) noncontractile tail. B40-8 is resistant to high pH values and tends to adsorb to solids (31). B40-8 does not replicate under the conditions in which filtration and elution experiments were done. Phages were assayed either by the double-agar-layer method (1, 4) or by an adaptation of the most probable number (MPN) method (32).

Sewage samples were collected in sterile glass bottles, transported to the laboratory under refrigeration, and analyzed within 4 h of sampling. Assays with the phage B40-8 were carried out with sewage to which the phage had been added at concentrations ranging from 1.6×10^3 to 6.1×10^5 per ml so that B40-8 outnumbered the other phages in the sample. After B40-8 addition, samples were gently shaken for 1 h at room temperature before being assayed.

To test the effect of membrane filter composition on the recovery of phages, 10-ml samples were passed through the following 0.22-μm (pore diameter) filters: Millex-GX (Millipore), with a polymeric membrane of cellulose esters (diameter, 25 mm; area of filtration, 4.9 cm^2); Millex-GV (Millipore) and Stirivex-GV (Millipore), with polymeric membranes of polyvinylidene difluoride (PVDF) and areas of filtration of 4.9 cm^2 (diameter, 25 mm) and 10 cm^2, respectively; and Anotep (Anotec Separations Ltd., Bambury, Oxon, England), with a membrane of inorganic material having a honeycomb pore structure and an area of filtration of 4.9 cm^2 (diameter, 25 mm). When indicated, 2 ml of 3% beef extract (Gibco) at pH 9.5 was passed through the filter before use as indicated elsewhere (25).

To elute phages retained in the membrane filters, 2 ml of 0.25 N glycine (pH 9.5) was passed through the membrane several times in both directions for 10 min, and the filtered wash fluid was then assayed for phages. Glycine has been shown to efficiently elute *B. fragilis* phages from solids (31). The numbers of phages were calculated by measuring the amount of filtered water. To elute phages associated to suspended solids in water, the pH of water samples was adjusted to 9.5 with 1.0 M glycine (pH 10.5) and the samples were gently shaken for 10 min at room temperature before filtration.

Considering existing data about the adequate cultural conditions for different strains of *B. fragilis* (2, 6, 7, 22, 23, 29, 34) and after assaying different compositions and conditions, we propose that the BPRM described above is a suitable medium in which to grow strain HSP40 of *B. fragilis* for bacteriophage assay. These culture conditions have advantages over others previously used (32). First, the duration of the lag phase never exceeds 3 h. Second, the doubling time is around 90 min, as compared to 200 min in

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TABLE 1. Effect of beef extract pretreatment on phage recovery with different types of membrane filters

Membrane filters	No. of expts	Ratio of pretreated to nonpretreated phage titer (mean ± SD)[a]
Cellulose esters	10	237 ± 222
PVDF		
Millex-GV	10	0.18 ± 0.31
Stirivex-GV	8	0.55 ± 0.64
Inorganic	8	0.60 ± 0.72

[a] These values represent the ratios of phage titers recovered after filtration of samples through pretreated filters to phage titers recovered after filtration through nonpretreated filters. The numbers of phages added to the natural samples always outnumbered the phages present in them and ranged from 1.6×10^3 to 6.1×10^5/ml. SD, standard deviation.

TABLE 2. Effect of elution on phage recovery with different membrane filters pretreated with beef extract or nonpretreated

Membrane filters	Beef extract pretreatment	No. of expts	Ratio of filtrate to eluate phage titer (mean ± SD)[a]
Cellulose esters	With	8	1.98 ± 2.21
	Without	8	1.66 ± 1.72
PVDF			
Millex-GV	With	8	0.44 ± 0.56
	Without	8	5.35 ± 3.04
Stirivex-GV	With	8	0.64 ± 0.50
	Without	8	3.21 ± 0.99
Inorganic	With	7	0.03 ± 0.03
	Without	7	0.43 ± 0.41

[a] Ratios are calculated as the phages titers recovered in the filtrate versus the phage titers recovered in the eluate. The titers in the eluate were calculated by measuring the volume of sample that passed through the filter. The numbers of phages added to the natural samples always outnumbered the phages present in them and ranged from 1.6×10^3 to 2.6×10^5/ml. SD, standard deviation.

MBB, and is similar to the best generation time described in the literature for *Bacteroides* species grown under strict anaerobic conditions (6, 7). Third, a microbial titer of 3.5×10^9 CFU/ml is obtained.

Frequently, variations in the growth medium change the efficiency of plating of a particular phage and also of phages in the environment (10, 13, 18). The use of BPRM permits the recovery of a slightly higher number of B40-8 phages than the use of MBB medium does. The addition of vancomycin and kanamycin to the assay medium did not significantly affect the efficiency of plating. The largest and most visible plaques were obtained when the bacteria used for direct plaque assay of phages came from a culture in the exponential phase containing around 3×10^8 bacteria per ml (optical density at 620 nm of 0.3 in this medium). Moreover, when we compare environmental data resulting from the use of BPRM versus MBB medium, data show that the use of BPRM permits the recovery of significantly (Student's t test, $P < 0.01$) greater numbers of phages than the use of MBB medium does, both with the direct assay method and with the multiple-tube test (see Table 3).

The enumeration of phages from environmental samples by using a rich medium, e.g., BPRM, even with antibiotics present in the assay medium, makes it advisable to decontaminate the sample. Filtration through a 0.22-μm filter is required to secure the absence of contaminating bacterial flora in the filtrates. Membrane filter composition affects phage recovery. Among those tested, PVDF membranes, either Millex-GV ($n = 5$) or Stirivex-GV ($n = 5$), allowed better recoveries (63.11% ± 15.0%) of the B40-8 phages added to sewage as indicated above than either cellulose esters ($n = 10$; 0.006% ± 0.008%) or inorganic membranes ($n = 10$; 0.13% ± 0.09%).

No generalizations can be made on the effect of pretreatment of membrane filters with 3% beef extract, pH 9.5 (Table 1). Pretreatment of cellulose ester membrane filters significantly increases B40-8 phage recovery in the filtrate, as described previously for coliphages (25). But pretreatment does not significantly improve performances of either PVDF or inorganic membrane filters. PVDF membrane filters provided the best recoveries under all conditions studied.

The results described above show that a number of B40-8 phages do not pass through the membrane filters, possibly because they adsorb to the filters or remain adsorbed to suspended solids that do not pass through the filters. Treatment of membrane filters with glycine after filtration of water samples resulted in the recovery of an additional number of phages. Phages were eluted from both beef-extract-pretreated and nonpretreated membrane filters. The numbers of eluted phages were of the same order of magnitude as those in the filtrate in all cases, except for inorganic filters pretreated with beef extract, where numbers of phages eluted significantly exceeded those in the filtrate (Table 2). In this case, the numbers of phages eluted approach the numbers of phages recovered after filtration through PVDF membrane filters.

Environmental phages infecting *B. fragilis* behave like B40-8 with respect to adsorption to membrane filters (Fig. 1). Accounting for phages recovered in the filtrate and in the eluate, PVDF membrane filters consistently gave significantly higher counts than the other two filters. In these sets of assays, all filters were pretreated with beef extract.

Because of the positive effect of an eluting treatment, we tried to elute phages adsorbed to suspended solids in water

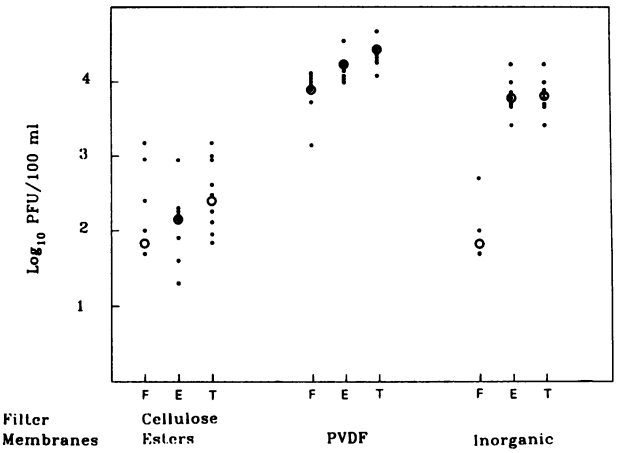

FIG. 1. Bacteriophages recovered from natural wastewater samples after membrane filtration through three kinds of membranes (cellulose esters, PVDF, and inorganic) and elution of phages from the filters. F, in the filtrate; E, eluted; T, F + E. Symbol: ○, mean value (geometric mean) of the set of assays.

TABLE 3. Recovery of phages of *B. fragilis* HSP40 from sewage by using the direct assay (PFU method) and the MPN method with MBB medium and BPRM[a]

Medium	Method of counting	Mean[b]	Range of counts (maximum to minimum)	No. of highest counts[c]
MBB	MPN	19	240 to <3	1[d]
	PFU	63	400 to <2	8[d]
BPR	MPN	660	3,900 to 93	6
	PFU	524	2,180 to 144	4

[a] These results are from 10 independent assays, and the values correspond to phages per 100-ml samples.
[b] Geometric mean.
[c] Expressed as the number of samples which gave the best recovery with each of the two methods.
[d] One value is 0 for the PFU method and <3 for the MPN method.

before filtration. However, our results (data not shown) indicate that the treatment of water with glycine before filtration does not significantly change the number of phages recovered. However, the possibility of phages adsorbed to suspended solids being released by other eluting treatments of water cannot be ruled out.

Kott (19), testing for coliphages, and Tartera (31), testing for phages of *B. fragilis*, found the multiple-tube test more efficient than the direct plaque assay test (PFU method) for the enumeration of phages in environmental samples. Other authors (5) report results which conflict with ours. Results presented herein show that phages recovered after filtration through and elution from PVDF membrane filters and counted by the PFU method do not differ significantly (Student's t test, $P < 0.01$) from the number recovered by using the multiple-tube test or MPN method (Table 3). Therefore, we suggest the use of the multiple-tube test only when large volumes of water with low phage contents need to be analyzed.

On the basis of the data reported herein, we recommend the use of PVFD membrane filters without beef extract pretreatment and the use of the double-agar-layer technique in BPRM for the recovery of phages infecting *B. fragilis* in environmental samples. Postfiltration elution of membrane filters may be advisable in samples with low phage contents.

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