Human Origin of *Bacteroides fragilis* Bacteriophages Present in the Environment

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*Bacteroides fragilis* HSP40 phages have been detected in waters with various levels of fecal contamination of human origin. The average numbers of *B. fragilis* phages present in sewage water reached $5.3 \times 10^9$ per 100 ml of water. We found a number 1,000 times lower in a river contaminated with domestic sewage only, in which the levels of fecal coliforms and fecal streptococci were 10,000 times lower than those found in raw sewage. In addition, *B. fragilis* phages were not found in significant numbers in slaughterhouse wastewaters. They were not present in fecal-polluted waters containing fecal contamination from wildlife only. Although the number of *B. fragilis* phages present in contaminated waters was lower than the number of coliphages, their presence indicated human fecal contamination. It is also shown that *Bacteroides* phages are only able to multiply under anaerobic conditions in the presence of nutrients, and they cannot multiply in natural waters and sediments.

Given the health risk that viruses represent, it is essential to develop more information on the nature and extent of viral contamination in the environment (2, 13, 14, 21). Ideally, isolation of the viruses themselves is the most appropriate means of virus detection. However, at this time, widespread direct testing for viruses is hampered by such factors as the long time required to obtain test results, variation in the precision and accuracy (i.e., detectability) of the different virus types, the shortage of competent personnel, and the high cost of viral analysis.

Consequently, it is desirable to establish, if possible, reliable indicator organisms and analytical methods to serve as surrogates for determining the presence of viruses. No universal indicator actually exists that is suitable for the detection of all viruses. Coliphages may have a restricted indicator function (5, 11, 18, 19). However, there are some unanswered questions on the ecological relationships between phages and their bacterial hosts once in the environment (15, 17). The potential use of *Bacteroides fragilis* bacteriophages as indicators of human enteric viruses has been explored (8, 20). Values of *B. fragilis* phages in human feces, sewage-polluted waters, and sediments have been shown (20). However, no data of other parameters of fecal pollution were obtained in the same samples. In this study we present data of phages infecting *B. fragilis* HSP40 in comparison with other bacteriological and virological parameters present in waters and polluted sediments, either with domestic sewage or with animal feces. Also, we report results showing that phages infecting *B. fragilis* do not replicate under experimental conditions that mimic the environmental ones. Together these results show that phages infecting *B. fragilis* HSP40 have the same origin as human viruses and they will not replicate significantly once in the environment.

### TABLE 1. Virological data of the samples analyzed

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>Range (mean) per 100 ml</th>
<th>% Positive samples</th>
<th>Range (mean) per 100 ml</th>
<th>% Positive samples</th>
<th>Range (mean) per 100 ml</th>
<th>% Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sewage (Prim)</td>
<td>20</td>
<td>$2.1 \times 10^2$-$4.6 \times 10^3$ (5.3 $\times 10^3$)</td>
<td>100</td>
<td>$1.5 \times 10^4$-$4.3 \times 10^6$ (1.2 $\times 10^6$)</td>
<td>100</td>
<td>0.0-$2.4 \times 10^9$ (257.8)</td>
<td>75</td>
</tr>
<tr>
<td>Raw sewage (Levante)</td>
<td>16</td>
<td>$2.3 \times 10^1$-$2.4 \times 10^3$ (1.3 $\times 10^3$)</td>
<td>100</td>
<td>$1.4 \times 10^4$-$2.4 \times 10^7$ (6.1 $\times 10^6$)</td>
<td>100</td>
<td>0.0-$2.4 \times 10^2$ (42.1)</td>
<td>75</td>
</tr>
<tr>
<td>Llobregat River</td>
<td>15</td>
<td>0.0-$43.0$ (6.7)</td>
<td>46</td>
<td>0.0-$4.6 \times 10^4$ (9.06 $\times 10^3$)</td>
<td>73</td>
<td>0.0-$0.04$ (0.0053)</td>
<td>13.3</td>
</tr>
<tr>
<td>Slaughterhouse 1</td>
<td>10</td>
<td>0.0-$1.2^a$</td>
<td>20</td>
<td>$1.0 \times 2.4 \times 10^4$ (9.06 $\times 10^3$)</td>
<td>100$^a$</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Slaughterhouse 2</td>
<td>10</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
<td>$0.0 \times 2.4 \times 10^3$ (5.3 $\times 10^3$)</td>
<td>100$^b$</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Water, Delta de l’Ebre</td>
<td>30</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
<td>$0.0 \times 69.0$ (7.2)</td>
<td>89</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Sediment, Delta de l’Ebre$^d$</td>
<td>30</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
<td>$0.0 \times 69.0$ (7.2)</td>
<td>89</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

$^a$ Value, <3 as determined by the MPN method and positive as determined by enrichment of 100 ml.
$^b$ Only positive or negative isolation by enrichment of 100 ml was determined.
$^c$ Cytopathic effect on BGM cells.
$^d$ Sediment values referred to 100 g of the sample.

* Corresponding author.
TABLE 2. Bacteriological data of the samples analyzed

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>Fecal coliforms per 100 ml</th>
<th>Fecal streptococci per 100 ml</th>
<th>C. perfringens per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Raw sewage (Prim)</td>
<td>20</td>
<td>7.2 x 10^5-1.6 x 10^6</td>
<td>2.7 x 10^7</td>
<td>2.0 x 10^6-6.4 x 10^7</td>
</tr>
<tr>
<td>Raw sewage (Levante)</td>
<td>16</td>
<td>4.9 x 10^5-4.7 x 10^7</td>
<td>1.6 x 10^7</td>
<td>1.1 x 10^6-4.4 x 10^6</td>
</tr>
<tr>
<td>Llobregat River</td>
<td>15</td>
<td>10.0-5.1 x 10^3</td>
<td>1.5 x 10^5</td>
<td>8.0-15 x 10^3</td>
</tr>
<tr>
<td>Slaughterhouse 1</td>
<td>10</td>
<td>3.4 x 10^5-4.6 x 10^5</td>
<td>2.3 x 10^6</td>
<td>1.0 x 10^5-1.1 x 10^6</td>
</tr>
<tr>
<td>Slaughterhouse 2</td>
<td>10</td>
<td>1.1 x 10^5-4.6 x 10^6</td>
<td>1.7 x 10^7</td>
<td>4.9 x 10^5-7.2 x 10^7</td>
</tr>
<tr>
<td>Water, Delta l'Ebre</td>
<td>30</td>
<td>0.0-1.5 x 10^6</td>
<td>73.5</td>
<td>0.0-4.6 x 10^6</td>
</tr>
<tr>
<td>Sediment, Delta l'Ebre</td>
<td>30</td>
<td>0.0-2.4 x 10^4</td>
<td>2.4 x 10^2</td>
<td>14.0-3.3 x 10^4</td>
</tr>
</tbody>
</table>

a Values referred to 100 g of sediment.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. B. fragilis HSP40 (20) was used for the quantification of bacteriophages and as the host strain in the replication experiments. Escherichia coli C600 (ATCC 23556) was used for the enumeration of coliphages.

Media and growth conditions. Modified blood agar base (MBAB) and modified brucella broth (MBB) (20) were used for the growth of B. fragilis HSP40. Soft agar was prepared by adding 5 g per liter of agar to MBB. MBAB-S (20) was used for the enumeration of B. fragilis phages in environmental samples.

When solid media were used, cultures were incubated in anaerobic jars (GasPak; BBL Microbiology Systems). Liquid cultures were grown in screw-capped tubes or bottles filled with MBB, avoiding the use of anaerobic jars. The temperature of incubation was 37°C.

Trypticase soy broth (TSB) and Trypticase soy agar (TSA) were used for the growth of E. coli C600. When enumerating coliphages, 0.12 g of MgSO₄·7H₂O and 0.05 g of CaCl₂ per liter were added to TSA and TSB media. Soft agar was prepared by adding 5 g per liter of agar to TSB.

Cell lines. A Buffalo green monkey continuous cell line (BGM) was used for the enumeration of enterovirus. BGM cells were grown in Eagle minimal essential medium with Earle's base, supplemented with 5% fetal bovine serum, 0.03% glutamine, 0.075% sodium bicarbonate, 100 U of penicillin per ml, and 100 mg of streptomycin per ml.

Virological and bacteriological assays. All bacteriophages in environmental samples were enumerated using an adaptation of the most-probable-number (MPN) method as described elsewhere (20).

Enteric viruses in sewage samples were enumerated without concentration of the sample. The other samples were concentrated using the glass powder adsorption-elution method (16).

Fecal coliforms and fecal streptococci were determined according to standard methods. Clostridium perfringens was enumerated using the method described by Handford (6).

Sampling sites and processing of samples. Raw sewage samples were collected from Colector Prim, which handles mainly domestic sewage, and Colector de Levante, which receives a mixture of domestic and industrial wastewater. Llobregat River samples were taken downstream from a domestic sewage source from a small mining town. Wastewater from two slaughterhouses was taken as samples containing high levels of animal fecal pollution. Water and sediments with low levels of fecal pollution from wildlife, and where no foci of human fecal pollution are known, were taken in the natural park Delta de l’Ebre, which receives a large number of migratory birds.

TABLE 3. Numbers of samples positive for coliphages, Bacteroides phages, and enteroviruses in different kinds of waters and sediments

<table>
<thead>
<tr>
<th>Sample, source</th>
<th>Organism</th>
<th>No. of samples with the following range of bacteria/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, lagoons with abundant wildlife</td>
<td>Coliphages</td>
<td>4/10 3/3</td>
</tr>
<tr>
<td></td>
<td>Bacteroides phages</td>
<td>0/10 0/3</td>
</tr>
<tr>
<td></td>
<td>Enteroviruses</td>
<td>0/10 0/3</td>
</tr>
<tr>
<td>Sediment, lagoons with abundant wildlife</td>
<td>Coliphages</td>
<td>11/12 4/4</td>
</tr>
<tr>
<td></td>
<td>Bacteroides phages</td>
<td>0/12 0/4</td>
</tr>
<tr>
<td></td>
<td>Enteroviruses</td>
<td>0/12 0/4</td>
</tr>
<tr>
<td>Wastewater, slaughterhouses</td>
<td>Coliphages</td>
<td>20/20</td>
</tr>
<tr>
<td></td>
<td>Bacteroides phages</td>
<td>2/20</td>
</tr>
<tr>
<td></td>
<td>Enteroviruses</td>
<td>0/20</td>
</tr>
<tr>
<td>Sewage-polluted waters</td>
<td>Coliphages</td>
<td>3/3 7/7</td>
</tr>
<tr>
<td></td>
<td>Bacteroides phages</td>
<td>3/3 4/7</td>
</tr>
<tr>
<td></td>
<td>Enteroviruses</td>
<td>1/3 1/7</td>
</tr>
</tbody>
</table>

a Range of numbers of bacteria per 100 ml of water or 100 g of sediment.
Water samples were collected in sterile glass bottles. All samples were placed at 4°C and examined within 6 h after collection. Sediment samples were always within 12 h after collection. Distilled water was placed in sterile plastic bottles. All samples were collected in sterile glass bottles. All samples were placed at 4°C and examined within 6 h after collection. Sediment samples were always within 12 h after collection.

### Results

#### Sample Preparation

Sediment samples were collected in sterile glass bottles. All samples were placed at 4°C and examined within 6 h after collection. Sediment samples were always within 12 h after collection. Distilled water was placed in sterile plastic bottles. All samples were collected in sterile glass bottles. All samples were placed at 4°C and examined within 6 h after collection. Sediment samples were always within 12 h after collection.

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Occurrence of bacteriophages in slaughterhouse wastewater. Wastewater from two slaughterhouses, containing high numbers of fecal bacteria (Table 2), was also tested for B. fragilis phages. Only 2 of 20 samples were positive following enrichment of 100 ml of wastewater (Table 1). Since no phages were detected by the MPN method, which detects a minimum of three phages per 100 ml, results suggest that a maximum of two phages per 100 ml were present in positive samples.

These bacteriophages could be either of animal origin or due to low input of human contamination. Anyway, the numbers are very small when compared with the values in domestic sewage (Table 1). In contrast, numbers of coliphages were nearly two logarithms higher than the numbers found in domestic sewage.

Cytopathic activity on BGM cells was noted in one of the samples. The virus isolated grew slowly as compared with the human enteroviruses able to grow in BGM cells. The isolated virus may be a nonhuman animal virus, like bovine reovirus, able to multiply in BGM cells.

Occurrence of phages in water and sediments from Delta de l'Ebre. A total of 60 samples (30 water samples and 30 sediments), containing a mean value of 73.5 fecal coliforms and 67.6 fecal streptococci per 100 ml of water and 2.4 x 10² fecal coliforms and 3.5 x 10² fecal streptococci per 100 g of sediment (Table 2), were tested for B. fragilis phages. All samples were negative for phages against B. fragilis HSP40 as well as for human enteroviruses (Table 1). Somatic coliphages were present in 55% of the water samples and in 89% of the sediment samples, although mean values were low: 3.4 per 100 ml and 7.2 per 100 g, respectively.

Numbers of coliphages and total coliforms were highest during the winter (data not shown). These data contradict previous reports on the presence of coliphages in natural environments (15). This may be due to the presence of a high number of migratory birds during the winter months. Contamination patterns throughout the year suggest that wildlife is the pollution agent.

Waters polluted by domestic sewage, with numbers of fecal coliforms and fecal streptococci similar to those found in the Delta de l'Ebre samples, were positive for the presence of Bacteroides phages (Table 3). The lack of phages recovered in samples collected in the national park is not imputable, then, to low levels of human fecal pollution.

Ratios among different microbial indicators of fecal pollution. The ratios among different bacterial and viral indicators enumerated in the work herein described are shown in Table 4. Ratios among coliphages and bacterial indicators differ clearly among the various samples studied. We are not able to detect differences between samples from domestic or animal origin.

The domestic sewage-contaminated samples containing Bacteroides phages and enterovirus showed a similar behavior of these two parameters, with respect to fecal coliforms and streptococci.

The greatest difference between Bacteroides phages and fecal coliforms and streptococci occurred in wastewater from slaughterhouses. This shows that even though phages

FIG. 1. Replication of phage B40-8 in natural water and sediment samples at different temperatures: (A) fresh water, 22°C; (B) fresh water, 30°C; (C) fresh water sediment, 22°C; (D) fresh water sediment, 30°C. Symbols: ■, anaerobic positive control (MBB medium was added to the water or sediment samples); □, B40-8 plus host bacteria in the environmental sample under anaerobiosis; ○, B40-8 plus host bacteria in the environmental sample under aerobiosis; ■, B40-8 in the environmental sample under aerobiosis.
isolated in these samples were of animal origin, it will still be possible to distinguish between human and animal contamination.

Replication of phages in natural environment. The ability of the bacteriophage B40-8 to replicate at either 22 or 30°C in freshwater and sediment samples in the presence of host bacteria was studied. No increase in phage number was observed under either aerobic or anaerobic conditions in any kind of environmental samples (Fig. 1). Only in the presence of necessary nutrients (MBB) for the host bacteria and under anaerobic conditions were the bacteriophages able to multiply.

DISCUSSION

Phages infecting B. fragilis HSP40 were found in waters polluted with domestic sewage, including waters with low levels of fecal contamination. In waters polluted with animal feces the phages were either not present or very scarce. When present, the ratio of fecal coliforms and fecal streptococci to B. fragilis phages was higher than 106, which does not compare to ratios found in domestic sewage or river water polluted with raw sewage. Ratios between B. fragilis phages and coliform bacteria in sewage were similar to the ratio existing in human feces, in agreement with the data of Kai et al. (9). According to the data available, the ratio between fecal coliforms and coliphages in sewage is at least 2 logs lower than in human feces (1, 5, 7, 9). Since the survival index, T99 (the time required for losses of 99% in the titer), of E. coli in sewage is high, these data may indicate that an important number of coliphages are rapidly originated outside the gut, either by lytic replication or by induction of lysogenic strains of E. coli. Bacteroides phages were also isolated from water samples contaminated with enteroviruses, and therefore both virus types seem to serve to differentiate human from animal fecal pollution. Male-specific coliphages, which have been proposed as potential indicators of animal fecal pollution (7), are always present in high numbers in samples polluted with animal feces. These data are in agreement with some preliminary results (data not shown). Published data (3, 9, 10, 20) show that the host range of phages infecting B. fragilis strains is very restricted. This could explain the ability of strain HSP40 to discriminate between human and nonhuman contamination. The reasons why the host range of B. fragilis phages is narrower than that of enterobacteria are not known. Both phages and host bacteria might have evolved more separately in obligate than in facultative anaerobes.

Numbers of coliphages were always much higher, by 2 or 3 logs, than those of phages infecting B. fragilis. The ratios between coliphages and fecal coliforms and between coliphages and fecal streptococci were quite variable in the different kinds of samples. These values ranged from 240 to 0.3 for fecal coliforms and 350 to 0.02 for fecal streptococci. These data are in concordance with results previously described (1). Results presented here show that coliphages did not allow differentiation of human from animal contamination, nor did male-specific coliphages. It seems that these phages probably could be used to differentiate gut-originated coliphages from those replicating in waters.

Bacteroides phages failed to replicate under optimal temperatures, adequate concentrations of host cells, and anaerobic conditions when added to environmental samples. Only under anaerobic conditions, with a high level of nutrients, and in the presence of host strain would the phage multiply. Although the possibility of finding these optimum conditions in the environment cannot totally be excluded, the replication of these bacteriophages outside the intestinal tract is very improbable.

The data herein presented show that Bacteroides phages do not replicate outside the intestinal tract and are capable of differentiating human from animal fecal contamination. Although additional work is needed, Bacteroides bacteriophages seem to be an excellent indicator of human viruses and may help to differentiate human from animal fecal contamination. Bacteriological methods available for this purpose are more complicated and slow (12).

ACKNOWLEDGMENTS

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LITERATURE CITED


