Enterophages, a group of phages infecting *Enterococcus faecalis*, and their potential as alternate indicators of human faecal contamination


**ABSTRACT**

We have developed a method for the detection of viruses in environmental samples that we have called enterophages, that specifically infect *Enterococcus faecalis*. This method has allowed us to determine the prevalence and to study the ecology of this group of phages. The enterophages replicate at 37°C, and at 41°C. The presence of NaNO₃ in the media inhibits the growth of background microbiota and allows an accurate, specific and rapid detection of these viruses. Enterophages were present in raw domestic sewage at lower concentrations (average $1.8 \times 10^2$ PFU/100 mL) than those of coliphages (average $1.7 \times 10^5$ PFU/100 mL). Phages were characterised by transmission electron microscopy showing icosahedral capsids, some with non-contractile tails as well as icosahedral non-tailed capsids. Different isolates had capsid sizes ranging from 20 nm to about 75 nm in diameter. These data describe a new group of phages that may serve as alternate indicators of human faecal pollution, especially in recreational waters. The ecology of these enterophages indicates that these may be strictly of human origin.

**Key words** | enterococci, enterophages, faecal contamination, indicators, recreational waters

**INTRODUCTION**

Pathogenic microorganisms are present in recreational waters and other water sources as a result of the presence of faeces of warm-blooded animals. Some examples of these pathogens are *Salmonella*, *Shigella*, *Campylobacter* spp., as well as several different types of enteric viruses and pathogenic protozoa. The low infectious dose (as low as 1 to 50 in some cases) of many of these pathogens is a cause for concern if they are released into the water environment, since humans who are in contact with these contaminated waters are bound to ingest the contaminated water, if there is primary contact. Human rotaviruses, astroviruses and noroviruses may be found in sewage-contaminated waters and in fact, among the waterborne pathogens, these are of greatest concern, since they may be more resistant to environmental conditions than pathogenic bacteria and they may be more resistant to chlorination and UV disinfection (Moe 2007), and have perhaps the lowest infectious dose of any of the waterborne pathogens.

Indicator organisms have been used for the last century as surrogates for the presence of pathogenic microorganisms in waters. In the past, bacterial indicators such as the thermotolerant coliform group, *Escherichia coli*, or the enterococcus group to determine the microbiological safety of waters have been tremendously useful. The levels of these indicators are monitored in treated drinking waters as well as recreational waters and the concentrations at which they are found enables (e.g.) beach managers in making decisions that involve closing beaches or keeping them open to the public.
Escherichia coli and Enterococcus spp. have been used as indicators of the presence of pathogenic microorganisms in different types of water. High concentrations of E. coli have been correlated with risk to bathers in freshwater beaches (Dufour 1984; Wheeler et al. 2002), whereas high concentrations of Enterococcus spp. have been correlated with risk in marine beaches. As such, they are now considered the most useful indicators of risk to bathers as a result of primary contact exposure to bathing waters. However, the above bacterial indicators have been isolated from pristine sites in a tropical rain forest (Rivera et al. 1988) and are routinely detected as part of the environmental microbiota in Puerto Rico and other tropical areas. Faecal indicators such as E. coli and enterococci may also be able to multiply and become a significant environmental source in tropical regions such as Hawaii, Guam, Puerto Rico and South Florida (Byappanahalli & Fujioka 2004). Enterococcus faecalis is useful because of its apparent inability to multiply in sewage contaminated waters, and its survival time in the environment seems to be greater than E. coli (Muniz et al. 1989). E. faecalis shows a closer correlation with risk to bathers in point-source impacted recreational waters (Dufour 1984); however, we know very little about the ecology of this group of microorganisms. Although E. faecalis has been described as having a limited host range, the genus may be more cosmopolitan than previously thought. The usual hosts include humans, chickens and dogs (Wheeler et al. 2002), but they are also present in the intestines of other animals, such as pigeons (Baele et al. 2002), and have also been found as part of the microbiota of insects, including flies (Martin & Mundt 1972; Macovei & Zurek 2006; Cox & Gilmore 2007). In Puerto Rico we have detected Enterococcus spp. as part of the microbiota in sand flies, and even in laboratory strains of Drosophila spp. (in preparation). Thus the role of the above bacteria as microbial indicators of faecal contamination is not entirely clear. New, reliable indicators of waterborne enteric viruses are needed.

Alternate microbial indicators of faecal water contamination

Thermotolerant and total coliforms tend to be poor indicator microorganisms because of their short survival and susceptibility to water treatments (Moe 2007). Moreover, these microbial indicators have the ability to grow in natural waters and there is a lack of correlation between the number of coliforms and that of infectious microorganisms (Toranzos et al. 2007). Also, pathogenic viruses have been found in waters where the number of coliforms had not exceeded the standards (Fong et al. 2004).

Bacteriophages, and specifically the coliphages (Stetler 1984), have also been used as model viruses for water quality control (IAWPRC 1991). Coliphages have been proposed as indicators of faecal water contamination (Havelaar & Hogeboom 1984; Loh et al. 1988; Hernandez-Delgado et al. 1991; Mocé-Llivina et al. 2005), as have those phages that infect Bacteroides fragilis (Tartera & Jofre 1987), since many have similar morphological characteristics to the enteric viruses as well as similar survival times in aquatic environments. However, their usefulness has been questioned, since the group is made up of different viruses belonging to several different families, each having different ecological and biological characteristics. A more specific group seems to be the male-specific RNA coliphages, but their usefulness is still being debated.

Out of all methods available for the determination of the microbiological water quality, the bacteriophage methods take the least amount of time, and are the most reliable methods for the detection of the target since they may not require confirmation procedures. Additionally, they may be amongst the least costly methods once they are implemented in an environmental monitoring laboratory.

The detection of pathogenic microorganisms or reliable indicator microorganisms is important for the determination of the microbiological quality of recreational waters. However, currently used detection methods have several disadvantages: they can be expensive or the detection time can take too long, taking on the average 24 to 48 h before results can be obtained. This is certainly not the best manner to manage possible risk in recreational waters. Additionally, bacterial indicators are not reliable indicators of the possible presence of enteric viruses. We need a method that will give reliable results in less than 12 to 18 h, and the only non-molecular method that seems to lend itself for this time interval is one that detects infectious bacteriophages.
Molecular methods are fast and reliable, but dead bacteria, naked DNA or non-infectious viruses may be detected, so the question of viability (in the case of bacteria) and infectivity (in the case of viruses) comes into play. Thus, the detection of infectious bacteriophages, when using the plaque assay, if they can be correlated to risk, would perhaps be the most appropriate manner of managing risk in recreational waters.

In the present paper we describe a method we developed for the detection of a group of phages that infect specifically Enterococcus faecalis.

MATERIALS AND METHODS

Host strains

Several enterococcal isolates were obtained from raw domestic sewage from several sewage treatment plants in Puerto Rico. These were obtained using membrane filtration using mEnterococcus media prepared as indicated by the manufacturer. Isolated colonies were purified and isolated in pure culture using the same media. Over 100 individual isolates were tested as possible hosts for the detection of phages in sewage using the single and double layer methods (Grabow & Coubrough 1986). Several Enterococcus spp. type strains were tested as possible phage hosts with raw sewage; these were: E. pseudoavium, E. faecium, E. durans, E. casseliflavus, E. hirae, E. dispar, E. gallinarum and E. faecalis. None tested positive for the presence of viral plaques, when using raw sewage as the inoculum, with the clear exception of Enterococcus faecalis. This bacterium was used in all subsequent experiments and was kept streaked for isolation in mEnterococcus agar plates and incubated overnight at 37°C. To prepare the host bacteria for plaque assays, individual colonies were picked and inoculated into sterile Dextrose Azide Broth medium and incubated overnight at 37°C. Five millilitres of this fresh culture was used for every 50 mL of sample added to 50 mL of 1X regular-strength Tryptic Soy Broth (TSB).

Samples

Sewage and recreational water samples were collected in sterile plastic bottles. Samples were shipped to the laboratory at 2°C to 8°C using cold packs and stored at 4°C. Additionally, bird, and several mammal fresh faecal samples were tested for the presence of enterophages.

Conditions for viral replication

Different concentrations of calcium chloride (CaCl₂·2H₂O Fisher Scientific Co. NJ) and sodium azide (NaN₃ MCB, OH) were tested, using as the base those concentrations used by other authors (Grabow & Coubrough 1986) for the detection of coliphages (in the case of calcium chloride) and commercially available mEnterococcus medium (in the case of sodium azide). CaCl₂ was tested at final concentrations of 0 mg/mL, 2.6 mg/mL, 5.2 mg/mL and 10.4 mg/mL. Similarly, NaN₃ was tested at 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL.

Several media were tested, including Nutrient Broth, Azide Dextrose, and mEnterococcus at different concentrations. Similarly, 2X concentration of Trypticase Soy Broth (TSB) and 1.5% agar was tested, as was regular-strength (1X) TSB containing different concentrations of agar. Agar concentrations ranging from 1.5% to 0.375% were tested. Plates were incubated at 22, 37, 41 and 45°C. Viral plaques were counted at 6, 24 and 48 h.

Single layer plaque assays

A 50 mL volume of 1X regular-strength Tryptic Soy Broth (TSB) was prepared and agar added to a final concentration of 0.75% or 0.375% when mixed with an equal volume of the sample. The medium was autoclaved and kept at 50°C. To a 50 mL volume of sample, CaCl₂ was added to a final concentration of 2.6 mg/mL, as was NaN₃, to a final concentration of 0.4 mg/mL plus 5 mL of a freshly grown culture (overnight culture) of E. faecalis. Solution was mixed carefully with the liquefied 50 mL volume of 1X TSB, and poured into sterile Petri dishes and then incubated at 22, 37 and 41°C, in order to determine if different phage populations that replicate at different temperatures are present. Viral plaques were counted at 6, 24 and 48 h of incubation.

Plaque isolation

Discrete viral plaques were plucked using standard procedures. Briefly, sterile glass Pasteur pipettes were used to
obtain a plug which was then placed in an Eppendorf tube containing 500 µL of sterile PBS. Many of these phages are tailed and rough treatment may break the tails rendering the viruses non-infective, thus the plug was gently dislodged and broken by pipetting up and down. The tubes were centrifuged at 14,000 rpm for 10 min at 10°C. The supernatant was transferred into a sterile Eppendorf tube, and the supernatant titred using serial dilutions using the double layer method using the same media as above. Briefly, the dilutions were mixed with 4 mL of 1X TSB containing the same concentrations of CaCl₂ and NaN₃ as above and 0.75% agar and then poured into Petri dishes containing 20 mL of bottom agar media containing CaCl₂ and NaN₃ and 1.5% agar. Plates were incubated for 24 h at 37°C. Then, for those plates showing complete viral lysis, 5 mL of sterile phosphate buffer saline (PBS) or physiological saline (0.85% NaCl) was added and slowly agitated rotationally for 20 min. The top agar was transferred to a sterile tube, pipetted up and down to break down the agar, and then centrifuged at 14,000 rpm for 10 min at 10°C. The supernatant containing the viruses was kept in a sterile tube at 4–7°C for titring prior to use.

**Transmission electron microscopy**

The resulting supernatant from above was loaded into dialysis tubing (12,000–14,000 MW cutoff, Spectrapor, Los Angeles, CA) for hydroextraction and covered with crystalline polyethylene glycol (PEG, Mol. Wt. 8,000, Sigma Chem. Co. MO; APHA 1998) and placed at 4–7°C overnight. The hydroextracted solution was recovered, the inside of the dialysis tubing washed with 100 µL of sterile 0.85% NaCl, transferred into a sterile Eppendorf tube and kept at 4°C till electronmicroscopic analyses.

An aliquot of the concentrated portion was placed on carbon Type-B 200 mesh copper grids or ultra thin carbon film/holesy carbon 400 mesh copper grids and stained with uranyl acetate (UA) 2%, pH 4.5 or potassium phosphotungstate (PTA) 2%, pH 7.2. Alternatively, 30 µL of each phage concentrate was placed on Parafilm and the grids floated on top for 15 minutes at room temperature prior to staining. All specimens were examined using a Karl Zeiss Leo 922 energy filtered transmission electron microscope operated at 200 KV. At least five phage particles of each type observed were measured. The phages were measured directly on the images, which had the magnifications previously calibrated. Capsid sizes were measured between opposite apices.

**Determination of burst sizes**

To determine the burst sizes of each phage isolate, *E. faecalis* was grown to mid-log phase in Azide Dextrose Broth (Difco Laboratories) and the concentration determined. One millilitre of the proper enterophage dilution was added in order to have a Multiplicity of Infection (MOI) of 1.0. The phage was allowed to come in contact with the bacteria by incubating at 35°C for 5 minutes. The solution was then centrifuged at 14,000 rpm at 10°C for 10 minutes. The supernatant was eliminated, and 10 mL of Azide Dextrose were added to the tube and the pellet resuspended to the original volume. Aliquots were obtained every 15 min for 3 h, and the aliquots serially diluted with 1X TSB containing the previously indicated concentrations of NaN₃ and CaCl₂. A volume of the resulting dilution was transferred into 4 mL of soft agar (0.375%) and poured into agar plates containing the bottom agar described above. The plates were incubated at 35°C for 24 h. Plaques were counted and burst sizes determined using the point in the graph where the values remained constant.

**Survival**

A sewage sample containing about 10⁷ enterophages/100 mL was inoculated into 1 L of unsterile sea water. A total of two litres of marine waters were used and separately incubated at 22 and 37°C. 100 mL aliquots were processed twice or thrice/week for 2 weeks using the single layer method as previously described. A total of eight petri dishes were obtained and incubated at 22, 37 and 41°C. Viral plaques were counted at 6, 24 and 48 h and concentrations were determined/100 mL.

**Prevalence**

Grab samples were obtained from the influent sites at several different sewage treatment plants in Puerto Rico and Montserrat. Samples were kept refrigerated till processing. Fifty-millilitre volumes were analysed as described above using the single-layer method.
RESULTS AND DISCUSSION

None of the Enterococcus spp. tested gave positive viral plaque results when using raw sewage as the inoculum. In fact several dozen Enterococcus spp. isolates were also tested, but only detected viral plaques when Enterococcus faecalis was used as the host microorganism. Media were also thought to be a variable important in the development of viral plaques and thus sewage and surface water grab samples were analysed using commercially available enterococci media (mEnterococcus, mE, mEI, Azide Dextrose), which did not result in any visible plaques using either the single or double layer method. This was surprising, since the media mentioned are standard for the isolation and detection of Enterococcus spp., but none were useful for the detection of viral plaques (data not shown). We also tested different concentrations of the TSB, and though we got some plaques, the growth of the host was not very conducive to the detection of viral plaques. When testing different concentrations of the media, we started out with 2X concentrations, since that is the standard procedure for the detection of coliphages; however, we were surprised to see that E. faecalis seems to be susceptible to the osmotic pressures created by even the final 1X resulting concentration after mixing with an equal volume of the sample. This phenomenon was even more obvious when testing marine waters, possibly as a result of the increased osmotic pressure; this opens up other questions as to the survivability of Enterococcus spp. in marine waters. Thus we decided to use a final concentration of 0.5% TSB. Though viral plaques were visible when using only 0.5% TSB under laboratory conditions, when processing environmental samples, it was impossible to see any plaques in the absence of NaN₃. Optimal plaque formation as well as host growth were observed at final concentrations of 2.6 mg/mL of CaCl₂ and 0.4 mg/mL of NaN₃. Viral plaques were easily seen at 0.75% agar at 37°C, but somehow clearer at 0.375% agar at 41°C.

We tested faecal samples from several different animals, including birds, dogs and cats as well as sand flies, for the presence of enterophages and none were positive; though all of them were positive for the presence of Enterococcus spp. (data not shown).

Viral morphology

The morphology of the virions (Figure 1) shows tailed phages and they are different from the round capsid morphology observed by others (Bachrach et al. 2003) in enterophages isolated from human saliva; though we also detected some isolates that were non-tailed, with icosahedral capsids about 20–25 nm in diameter (data not shown). Our best studied phage isolate to date showed an icosahedral 75 nm diameter capsid with a long non-contractile tail measuring 240 nm long by 15 nm wide. This morphology is similar to the classical Bradley’s basic morphology Group B belonging to the Siphoviridae family (Matthews 1982). Further studies are needed to characterise other groups of enterophage isolates. It should be noted that viruses infecting E. faecalis have been described previously, but not those from environmental samples. Previous isolation and use of these phages was for typing of E. faecalis clinical isolates (Pleceas & Brandis 1974) going back several decades. The fact that enterophages are also found in human saliva (Bachrach et al. 2005) may indicate that they are in fact unique to humans, and, as humans usually spit when they immerse their heads into the water while swimming, the concentrations of these enterophages may prove to be a good indicator of risk to bathers.

Burst sizes

When viruses successfully infect cells, this results in a certain number of progeny virions called the burst size. It would seem apparent that the phages most likely to be
found in sewage and fecally-contaminated waters are those that have a large burst size. Sewage isolates were found to have different burst sizes ranging from 10 to 1,000 virions/cell (data not shown); thus, as expected, the burst size varies depending on the viral isolate. We are focusing our characterisation efforts on those enterophages that have the largest burst sizes, since they may be more likely to be detected in low volumes of environmental water samples than those viruses with a small burst size; however, this will depend on the ecology of each group and their prevalence world-wide.

Survival

Any indicator of risk should be at least as reliable as those currently in use and should meet several criteria, and among these the ability to survive for at least as long as the pathogens under the environmental conditions is a must. Our data (Figure 2) show that there may be different populations of enterophages present in raw sewage, as indicated by the different numbers obtained in these experiments in plates incubated at three different temperatures. However, all three populations survived similarly and their numbers decreased by over 2 log 10 over a period of 7 days, which is similar to the survival times of enteric viruses as reported by others (Ward et al. 1986). Figure 2(A) shows that the enterophages survived better at ambient temperature where they showed about a 0.5 log 10 drop after 3 days, whereas there was at least 1–1.5 log 10 drop in concentrations at 37°C after the same length of time. It should be noted that there was no replication of the enterophages at ambient temperature in spite of having native E. faecalis present in the sewage inoculum which would have been shown by an increase in the number of viruses. It is also noteworthy to see different groups of enterophages capable of replicating at different temperatures (namely 22, 37 and 41°C). These data will guide future studies as to the types of enterophages to focus on as the possible best indicators of human faecal contamination. We are in the process of carrying out similar survival experiments in marine waters, freshwaters, beach sand and soils in the presence of the E. faecalis host we have been using.

Prevalence

Coliphages and enterophages in treated and untreated sewage from Puerto Rico were present at concentrations ranging from $1.4 \times 10^5$ to $2.6 \times 10^5$ and from 55 to 363 Plaque Forming Units or PFU/100 mL, respectively (Tables 1 and 2). It is also noteworthy that the enterophage plaques could be easily detected even in raw sewage samples after 8 h. Coliphage analyses of the same samples showed luxuriant growth of background microbiota that masked the viral plaques; even in the presence of antibiotics.

Table 1  | Concentrations of coliphages in sewage treatment plants in Puerto Rico

<table>
<thead>
<tr>
<th>Sewage treatment plant</th>
<th>Influent (PFU/100 mL)</th>
<th>Effluent (PFU/100 mL)</th>
<th>% of removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerto Nuevo</td>
<td>$2.6 \times 10^5$</td>
<td>$1.6 \times 10^4$</td>
<td>94.0</td>
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<tr>
<td>Carolina</td>
<td>$1.4 \times 10^5$</td>
<td>$3.5 \times 10^3$</td>
<td>98.0</td>
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<tr>
<td>Bayamón</td>
<td>$1.5 \times 10^5$</td>
<td>$1.4 \times 10^3$</td>
<td>&gt;99.0</td>
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<tr>
<td>Caguas</td>
<td>$1.4 \times 10^5$</td>
<td>ND</td>
<td>&gt;99.0</td>
</tr>
</tbody>
</table>

ND-Not detected.
These data also showed that sewage treatment plants are able of removing/inactivating from 87% to >99.9% of influent enterophage concentrations, and the removal/inactivation of coliphages was always >90% in the same samples.

### CONCLUSIONS

In conclusion, we describe a method developed for the detection of enterophages in environmental samples. This method could lend itself to any type of sample such as water, sewage, soils, food and sand. We have also detected these phages in beach sand samples, but are working on an elution procedure that may also be used for food samples. These viruses need to be studied in order to determine if, in fact, they can be used as alternate indicators of human faecal contamination. We urge those readers who may try to determine the prevalence of enterophages under their own conditions to try to use the same procedures as well as the same host in order to be able to compare results in the future. One of the problems we have encountered with coliphages is that many different hosts were used over the years, making it difficult to determine if in fact the same coliphages are being detected in different geographical areas of the world. We hope to avoid this problem with this group of viruses, and to achieve this goal we have started an international collaboration with colleagues from several different geographical areas encompassing several continents, to determine the prevalence of enterophages in raw sewage and recreational waters. This will let us know if these phages can be used as alternate indicators of human faecal contamination in waters and other media at a global scale. Further work also needs to be done to further characterise enterophages not only morphologically, but also molecularly before they are taken into consideration as indicators of human faecal contamination. The molecular characterisation will undoubtedly result in the development of rapid methods, such as the polymerase chain reaction for their detection in recreational and other types of waters and matrices.

### ACKNOWLEDGEMENTS

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### REFERENCES


### Table 2

<table>
<thead>
<tr>
<th>Sewage treatment plant</th>
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<th>Effluent (PFU/100 mL)</th>
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