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Bacteriophage Lysis of *Enterococcus* Host Strains: A Tool for Microbial Source Tracking?

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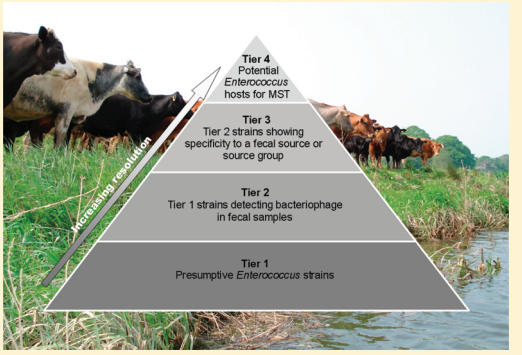
ABSTRACT: This paper describes the isolation of *Enterococcus* host strains, for potential use as simple bacteriophage (phage)-based microbial source tracking (MST) tools. Presumptive *Enterococcus* host strains were isolated from cattle feces, raw municipal wastewater, agricultural runoff, and waters impacted by farms or wastewater treatment works (WWTW) in southern England, United Kingdom (UK). All enterococcal host strains ($n = 390$) were first screened for their ability to detect phage in samples of raw municipal wastewater and fecal material from cattle, pigs, and sheep. Host strains that detected phage ($n = 147$) were ranked according to both their specificity to a particular fecal source and also the number of phages (expressed as plaque-forming units, PFU) that they detected per milliliter of sample. Host strains that demonstrated host specificity and which detected phages at levels greater than 100 PFU/mL ($n = 29$) were further tested using additional fecal samples of human and nonhuman origin. The specificity and sensitivity of the enterococcal host strains were found to vary, ranging from 44 to 100% and from 17 to 83%, respectively. Most notably, seven strains exhibited 100% specificity to either cattle, human, or pig samples. Isolates exhibiting specificity to cattle were identified as belonging to the species *Enterococcus casseliflavus*, *Enterococcus mundtii*, or *Enterococcus gallinarum*, while human and pig isolates were members of either *Enterococcus faecium* or *Enterococcus faecalis*. The high specificity of phages infecting *Enterococcus* hosts and the simplicity and relatively low cost of the approach collectively indicate a strong potential for using this method as a tool in MST.



INTRODUCTION

Contamination of surface waters with feces of human and nonhuman (domestic, agricultural, and wild animal) origin leads to increased public health risk of exposure to pathogens through drinking water supply, aquaculture, and recreational activities.[1−3] Of the two, human fecal contamination is considered to be the greater risk to public health, largely because viruses that commonly cause illness are highly host specific, but feces from nonhuman sources also pose a potential risk of infection by zoonotic pathogens, such as *Escherichia coli* O157, *Giardia* spp., *Campylobacter* spp., and *Cryptosporidium* spp.[4] Recent research findings have suggested that the risk to human health attributable to contamination by nonhuman fecal sources is varied and, specifically, that the risk to human health from fresh cattle feces in recreational waters is not substantially different than that from human fecal contamination.[5]

The Clean Water Act (CWA) and the Water Framework Directive (WFD) regulate surface water quality standards in the United States (US) and the European Union (EU), respectively.[6,7] These legislative measures require the identification and management of those point and diffuse sources of microbial pollution that lead to the impairment (US) and noncompliance (EU) of surface waters. In the US, impaired waters are investigated and actions for remediation are set out by the principle of "total maximum daily loads" (TMDL). TMDL define the quantity of pollutant that a water body can tolerate while adhering to water quality standards.[6] Similarly, the WFD in the EU requires the establishment of a "program of measures" for identified river basin districts, in order to achieve a "good status" by 2015.[7] Water quality standards are monitored using fecal indicator organisms (FIO), typically intestinal enterococci and *E. coli*, as it remains technically complicated and expensive to monitor pathogens directly.[8] FIO concentrations offer an indication of the presence of fecal contamination and the potential risk to public health, but they do not determine contamination sources. Since certain fecal sources pose a greater risk to human health than others, reliance on FIO concentrations alone could result in either an underestimation or overestimation of the potential risk. Legislative requirements, the need to predict risk to human health, and the implications of source on the selection of appropriate remediation measures, have led to the development of the field of microbial source tracking (MST). MST encompasses techniques that aim to distinguish source(s) of fecal contamination in surface and ground waters. MST is a rapidly advancing field, but there is currently no single standardized method available to distinguish sources of fecal contamination in surface waters in all situations.[9,10]

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Simple, low-cost, and effective phage-based MST techniques involving the detection and enumeration of phages infecting *Bacteroides* spp. and the subgroups of F-specific RNA phage have been used to discriminate human from nonhuman fecal contamination.[11,12] Recent research has shown that the inactivation profiles of F-specific RNA phage subgroups differ, with phages of groups III and IV being less resistant to inactivation by natural stressors, thus hindering the interpretation of results when using F-specific phage in MST.[13] Host strains of *Bacteroides* spp. that are demonstrably restricted to human and nonhuman feces have been isolated from different geographical regions[14−17] and have been used successfully to distinguish human and nonhuman fecal contamination of surface waters. An advantage of detecting bacteriophage (phage) of *Bacteroides* spp., as opposed to a phage infecting aerobic or facultatively anaerobic hosts such as *E. coli* (somatic coliphage), is that the host bacterium requires strict anaerobic conditions and is therefore unlikely to replicate outside the gut environment. However, recent research into the ecology of somatic coliphage, suggests that any replication of their bacterial host in the natural environment is unlikely to have a significant impact on the numbers of phage detected.[18] With this in mind, it may be advantageous to use an alternative host genus that does not need to be handled under strict anaerobic conditions and which has simpler growth requirements. The intestinal enterococci are an "indicator of choice" in many parts of the world for determining water quality, and phages capable of infecting *Enterococcus faecalis* have already been proposed as a potential alternative indicator of human fecal contamination.[19] Phage-based MST methods have tended to focus on identifying human fecal pollution, but the need to identify diffuse nonhuman fecal contamination in order to meet the stipulations of the CWA and WFD is clear. The objectives of this study were therefore (1) to evaluate the ability of phages infecting *Enterococcus* host strains to identify human and specific nonhuman sources of fecal contamination and (2) to establish an effective protocol for isolating new *Enterococcus* host strains suitable for MST application in other parts of the world.

MATERIALS AND METHODS

Sample Collection. Samples of animal feces, cattle and pig runoff, wastewater, and surface water were collected for the isolation of potential *Enterococcus* host strains and for subsequent bacteriophage detection. Fecal material from either pooled individual scats (at least 20 individuals) or agricultural runoff was collected from cattle, ducks, geese, goats, horses, pigs, rabbits, seagulls, and sheep on 25 occasions, from 2008 to 2010, using sterile swabs and 100 mL sampling containers (Nalgene) as appropriate. Previous research has shown that not all individuals in a population will excrete selected phage and that the titers may vary,[20] so to obtain a sample representative of a population, all individual scat samples were pooled so that they included feces from at least 20 different animals.

In the laboratory, scat samples were mixed and homogenized using a Seward Stomacher 400 (Lab System) in sterile one-quarter strength Ringer's solution (Fisher Scientific). Fecally contaminated runoff water was collected from cattle housing and from the drainage pipes of pig housing at Wales Farm, Plumpton Agricultural College, in East Sussex, UK. Samples of wastewater (raw and treated) were collected from seven biological wastewater treatment works (WWTW) situated in South East England, UK. Population equivalents of the sites ranged from 258 to 53 425. Surface water samples were collected downstream of two livestock farms (Wales Farm and Pellingbridge Farm) and downstream of a medium sized WWTW (population equivalent, 55 955). All surface water and wastewater samples were collected in 1 L sterile sample bottles (Nalgene) using an extendable sampling pole, from approximately 30 cm below the surface of the water. Following collection, samples were transported to the laboratory in the dark at 4 °C within 4 h. Samples collected for the isolation of enterococci were processed immediately, whereas samples for phage enumeration were preserved with 10% glycerol at −20 °C[21] for analysis within 4 weeks.

Isolation of Presumptive *Enterococcus* Host Strains. Potential host strains were obtained from pooled individual cattle scats (approximately 25), cattle and pig fecal runoff, municipal wastewater, and surface waters impacted by human and nonhuman sources of feces in southern England, UK, and a dilution series was prepared. These samples were then passed through 0.45 μm membrane filters for enumeration of presumptive enterococci in accordance with ISO 7899/2.[22] m-Enterococcus Agar (Becton Dickinson Microbiology Systems) was used as a selective medium for the isolation of presumptive enterococci and was incubated at 37 °C (±2 °C) for 44 h (±2 h). Following incubation, filter membranes (Thermo Scientific Nalgene) with between 10 and 60 colony forming units (CFU) were transferred to prewarmed bile esculin agar (Oxoid) plates and incubated for a further 4 h at 44 °C. Distinct colonies demonstrating esculin hydrolysis (blackening of the media) were picked and streaked onto m-Enterococcus Agar in order to obtain pure cultures. Further identification was achieved by undertaking catalase and Gram stain tests (in which morphology was also recorded). Presumptive *Enterococcus* host strains (Gram positive, coccoid, catalase negative, esculin positive) were then grown at 37 °C (±2 °C) for 24 h (±2 h) in tryptone soya broth (TSB) (Oxoid), mixed with 50% glycerol, and preserved at −80 °C for up to 6 months prior to further testing.

Bacteriophage Enumeration. Phages infecting *Enterococcus* hosts were enumerated using a previously described and ISO standardized double-agar-layer[23] method, and the results were expressed as plaque-forming units (PFU) per 100 mL of sample. Different media were tested for the top and bottom agar layers, including m-Enterococcus Agar, nutrient agar, KF Streptococcus Agar, and tryptone soya agar (TSA) (Oxoid). The clearest plaques were obtained using TSA for both layers, so TSA was therefore used for all subsequent phage assays. This observation is in accordance with results from a previous study that focused on phage of *E. faecalis*.[19] The concentrations of agar in top and bottom layers used were the same as those for standardized methods reported elsewhere (ISO 10705/2).[24]

Homogenized fecal samples were diluted (1:10 w/v) with one-quarter strength Ringer's solution and centrifuged at 3000*g* for 10 min. The resulting supernatants were filtered through 0.22 μm polyvinylidene diflouride membrane syringe filter units (Millipore). A 1 mL portion of each sample was added to 1 mL of exponentially growing host strain and 2.5 mL of tryptone semisolid agar. The resulting suspension was mixed briefly using a Whirlimixer (Fisher Scientific) and poured onto previously prepared TSA bottom agar layers in 90 mm diameter Petri plates. Once solidified, plates were inverted and incubated at 37 °C (±2 °C) for 18−24 h. All fecal samples were tested in triplicate. Clearly visible circular "zones of lysis" in a confluent lawn of enterococcal host were recorded as PFU (Figure 1).

Isolate Screening. A tiered approach was designed and implemented in order to reduce in a rational manner the initial large

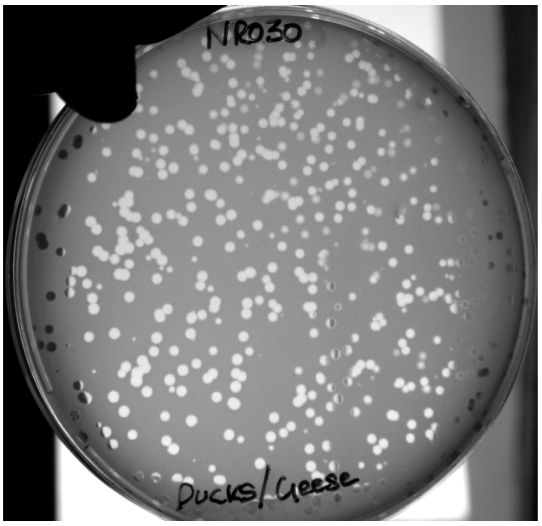

Figure 1. Enterococcal host strain Newhaven River Ouse 30 (NRO30), demonstrating clear plaques from a duck and goose mixed fecal sample.

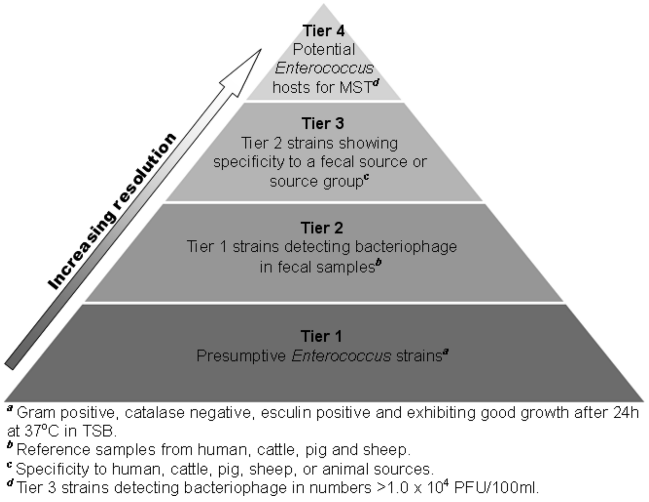

Figure 2. Tiered approach to the isolation of *Enterococcus* host strains.

number of enterococcal hosts to a smaller subgroup that would be suitable for phage enumeration and MST (Figure 2). This approach provided a protocol that could rapidly eliminate enterococcal strains that would not be effective hosts and focus efforts on those host strains warranting further investigation.

Tier 1 hosts were those strains confirmed as being presumptive *Enterococcus* spp. that grew well at 37 °C (±2 °C) for 24 h (±2 h) in TSB. All tier 1 host strains were screened against the same battery of reference samples containing phage from seven municipal wastewater samples and five pooled cattle, sheep, and pig fecal samples. Those enterococcal strains that detected one or more PFU per 100 mL (tier 2 hosts) were ranked according to their specificity (to a particular source) and to the numbers of phage that they detected (PFU/100 mL). Specificity was given a higher weighting, as this characteristic was considered essential for MST application. It was important that the phage infecting the hosts produced clear well-defined plaques (Figure 1). Hosts that demonstrated plaques that were unclear, and therefore difficult to identify and enumerate, were not assessed further. Tier 2 hosts that showed specificity to a particular fecal source or source group (tier 3 hosts) and which also detected phage in greater numbers than 100 PFU per 100 mL (tier 4 hosts) were considered to be the host stains with the greatest potential for MST application. Host strains detecting higher concentrations of phage in fecal samples (tier 4 host strains) have greater potential for MST application, because they are more likely to detect phage in surface waters, where phage are likely to be present in much lower numbers. All 25 tier 4 hosts, as well as four tier 3 hosts demonstrating 100% specificity and clear plaque production, were then subjected to further rigorous testing using inputs representative of those present across the whole study catchment.

Host Identification. Host strains meeting the criteria for specificity, sensitivity, and plaque clarity were identified to species level. This process was carried out using the API 20 Strep identification system (BioMérieux) according to the manufacturer's instructions, and a six step biochemical key described by Manero and Blanch.[25] To aid identification using API 20 Strep, tests for yellow pigmentation were also performed. Production of yellow pigmentation was demonstrated by growing the enterococcal hosts on nutrient agar and incubating for 24 h at 37 °C. Following incubation, host strains were checked for yellow pigmentation against a white filter paper.

Statistical Analysis. Performance of the enterococcal strains as potential hosts for MST was evaluated in relation to their specificity and sensitivity to a particular fecal source. Specificity and sensitivity were calculated using standard definitions[26] and expressed as a percentage.

The Spearman's rank correlation coefficient was used to test the hypothesis that a negative relationship between specificity and sensitivity existed. The Kruskal−Wallis test was used to determine variation of specificity and phage numbers detected on host strains obtained from different sources. Where variation was significant, post hoc Mann−Whitney tests were used to determine where variation occurred. Statistical tests were performed using the statistical package SPSS 16.0, with the significance level set at 5%.

RESULTS

Host Strain Screening. In total, 554 potential enterococcal hosts were isolated, and 390 were confirmed as tier 1 hosts (Table 1). Thirty-eight percent of hosts detected phage in reference samples from cattle, pig, sheep, and raw municipal wastewater (tier 2 hosts). A high percentage of host strains (67% and 68%, respectively) isolated from cattle and pig runoff detected phage in reference samples, but far fewer host strains isolated from both municipal wastewater and surface waters (34% and 35%, respectively) and only one out of 76 hosts obtained from pooled cattle scats detected phage in any of the four reference samples. A sum of 117 tier 2 hosts was restricted to one fecal source or source group (tier 3 hosts) and 25 tier 3 hosts detected phages in numbers greater than 1.0×10^4 per 100 mL of sample (tier 4 hosts). Sixty-eight percent of tier 4 hosts originated from cattle runoff, 24% from surface waters, 4% from pig runoff, and 4% from raw municipal wastewater. Finally, 25 tier 4 hosts and four additional tier 3 hosts (investigated further because they had 100% specificity and excellent plaque clarity) underwent further investigation, and of the 29 enterococcal hosts, just over 48% were found to be highly specific to a particular source or source group. Our screening process therefore offers a rapid

Table 1. Assignment of Potential *Enterococcus* Host Strains from Various Sources According to the Four Different Tier Categories

		no. of strains			
host origin	no. of samples	tier 1[a]	tier 2[b](%)	tier 3[c] (%)	tier 4[d] (%)
pooled fecal scats from cattle	1	76	1 (1)	1 (1)	0 (0)
liquid runoff from cattle	2	83	56 (67)	52 (63)	17 (20)
liquid runoff from pig	1	31	21 (68)	12 (39)	1 (3)
municipal wastewater	1	112	38 (34)	29 (26)	1 (1)
impacted surface waters	3	88	31 (35)	23 (26)	6 (7)
total	8	390	147 (38)	117 (30)	25 (6)

[a] Tier 1: presumptive *Enterococcus* (Gram positive, catalase negative, esculin positive, and exhibiting good growth after 24 h at 37 °C in TSB). [b] Tier 2: tier 1 strains that detect bacteriophage >0 PFU/mL in human, cattle, pig, or sheep reference fecal samples. [c] Tier 3: tier 2 strains that show potential specificity to human, cattle, pig, sheep, or animal reference fecal samples. [d] Tier 4: tier 3 strains detecting bacteriophage in reference fecal samples >100 PFU/mL.

Table 2. Number of Plaque Forming Units (PFU/100 mL) Detected by Tier 4 Presumptive *Enterococcus* Host Strains in Pooled Fecal Samples from Different Origins

		PFU/100 mL for samples of different origins (no. of samples)			
host origin	host ID	municipal wastewater (n = 7)	cattle runoff (n = 5)	pig runoff (n = 5)	sheep feces (n = 5)
cattle runoff	CR1	0	1.7×10^4	0	0
cattle runoff	CR4	0	1.4×10^4	0	0
cattle runoff	CR6	0	2.1×10^4	0	0
cattle runoff	CR7	0	1.3×10^4	0	0
cattle runoff	CR15	0	8.3×10^4	0	0
cattle runoff	CR16	0	4.4×10^4	0	0
cattle runoff	CR17	0	7.1×10^4	0	0
cattle runoff	CR37	0	1.2×10^5	0	0
cattle runoff	CR45	0	1.8×10^4	6.0×10^2	0
cattle runoff	CR47	0	4.8×10^4	1.2×10^3	0
cattle runoff	CR51	0	5.2×10^4	2.0×10^3	0
cattle runoff	CR58	0	1.9×10^4	0	2.0×10^2
cattle runoff	CR61	0	2.2×10^4	1.7×10^3	0
cattle runoff	CR63	0	3.4×10^4	0	0
cattle runoff	CR70	0	7.3×10^4	1.0×10^3	0
cattle runoff	CR73	0	2.3×10^4	2.1×10^3	0
cattle runoff	CR75	0	4.8×10^4	1.0×10^2	3.0×10^2
municipal wastewater	MW42[a]	2.7×10^3	0	0	0
municipal wastewater	MW47[a]	3.4×10^3	0	0	0
municipal wastewater	MW96	0	0	$>2.0 \times 10^5$	0
Newhaven (River Ouse)	NRO5[a]	0	0	9.8×10^3	0
Newhaven (River Ouse)	NRO8[a]	3.4×10^3	0	0	0
Newhaven (River Ouse)	NRO20	0	1.0×10^5	0	0
Newhaven (River Ouse)	NRO24	0	0	1.1×10^5	0
Newhaven (River Ouse)	NRO30	0	1.2×10^4	0	0
Newhaven (River Ouse)	NRO39	0	2.5×10^4	0	0
pig runoff	PR3	0	2.2×10^4	1.0×10^2	0
Pellingbridge (River Ouse)	PRO4	0	2.0×10^4	0	0
Wales Farm stream	WFS7	0	6.2×10^4	0	0

[a] Tier 3 host strains with 100% specificity and excellent plaque clarity, but they detected lower plaque counts ($<1.0 \times 10^4$) than tier 4 host strains

approach to determining the suitability of potential hosts for MST application.

Specificity vs Sensitivity. Following initial screening, 29 host strains emerged as potentially suitable for MST application (Table 2). The suitability of these enterococcal hosts was further tested by exposing them to 37 additional samples from 10 source groups (Table 3). The specificity of the host strains ranged from 44 to 100%. For MST purposes it is particularly important that the hosts do not detect phage from both human and animal sources (cross-reaction). Further testing revealed that 15 of the

Table 3. Percentage of Phage Positive Samples from Different Origins Detected Using Four *Enterococcus* Host Strains

		% phage positive samples from *Enterococcus* host strains			
sample origin	no. of samples	municipal wastewater 47	cattle run-off 70	Wales Farm stream 7	Newhaven River Ouse 24
cattle	15	0	67	33	0
chicken	6	0	0	0	0
ducks and geese	6	0	100	0	0
goat	6	0	0	0	0
horse	6	0	0	0	0
pig	15	0	100	0	20
rabbit	6	0	0	0	0
seagull	6	0	0	0	0
sheep	6	0	0	0	0
raw MW[a]	15	100	0	0	0
final MW[a]	12	25	0	0	0

[a] MW = municipal wastewater.

Table 4. Identification of *Enterococcus* Hosts Strains with API 20 Strep and a Biochemical Key

host origin	ID	API 20 Strep identification	biochemical key identification
cattle runoff	CR1	*E. casseliflavus*	*E. mundtii*
cattle runoff	CR6	CNI[a]	*E. gallinarum*
cattle runoff	CR7	*E. casseliflavus*	*E. casseliflavus*
cattle runoff	CR15	*E. casseliflavus*	*E. casseliflavus*
cattle runoff	CR16	CNI[a]	*E. gallinarum*
cattle runoff	CR17	CNI[a]	*E. gallinarum*
cattle runoff	CR70	*E. gallinarum*	*E. gallinarum*
municipal wastewater	MW42	*E. faecalis*	*E. faecalis*
municipal wastewater	MW47	*E. faecium*	*E. faecium*
Newhaven (River Ouse)	NRO8	*E. faecalis*	*E. faecalis*
Newhaven (River Ouse)	NRO24	*E. faecium*	*E. faecium*
Newhaven (River Ouse)	NRO30	*E. casseliflavus*	*E. casseliflavus*
Newhaven (River Ouse)	NRO39	*E. gallinarum*	*E. faecium*
Wales Farm stream	WFS7	*E. gallinarum*	*E. gallinarum*

[a] CNI = API 20 Strep could not identify.

hosts cross-reacted with animal feces and human wastewater samples, even though they had previously been restricted to either human or nonhuman sources. This ruled them out from any further analysis. At this stage, 14 potential hosts remained specific to a single human or nonhuman source. Notably, seven host strains were 100% specific to a single source (e.g., cattle, pig, or human). Table 3 illustrates the results for four host strains specific to either cattle, pig, human, or mixed fecal sources. Strains WFS7 and NRO24 isolated from surface waters were 100% specific to cattle and pig fecal samples, respectively. However, the sensitivities of WFS7 and NRO24 were only 33% and 20%, respectively, and, interestingly, they appeared to be restricted to samples originating from specific herds present on Wales Farm, Plumpton, UK. Human specific host strain MW47 had a much higher sensitivity, detecting phage in all raw wastewater samples (100%) tested from six WWTW, one of which has a population equivalent of only 258. However, MW47 was only detected in one-fourth of the treated wastewaters tested, suggesting removal or die-off of phage during treatment. Certain host strains such as strain CR70, while demonstrating lower levels of phage specificity (Table 3), had sensitivity levels much higher than more specific hosts. Strain CR70 was found almost exclusively in cattle and pig samples (90% specificity), had a sensitivity of 83%, and could be useful as an indicator of nonhuman fecal contamination.

Spearman's correlation coefficient was used to test the relationship between specificity and sensitivity of the 29 promising enterococcal hosts. Results showed a moderate negative relationship between sensitivity and specificity ($R_s = -0.480$, $p < 0.01$). As specificity increased, the sensitivities of the hosts also tended to decrease. Ideally, the specificity and sensitivity of a host for phage lysis would be 100% in each case.

Host Strain Origin. As shown in Table 1, potentially useful enterococcal hosts were successfully isolated from five samples originating from a range of sources. In order to analyze variations in host specificity and sensitivity, all enterococcal hosts were classified as being members of one of four source groups [(1) pooled cattle feces, (2) cattle and pig fecal runoff, (3) municipal wastewater, or (4) surface waters]. The Kruskal−Wallis test suggested that specificity did not vary significantly between host strains isolated from the different source groups ($p > 0.05$), though statistically significant variations in the number of phage

in each source group were apparent ($p < 0.01$). Post hoc Mann−Whitney tests revealed that numbers of phage capable of infecting host strains isolated from cattle feces were significantly lower when compared to numbers of phage infecting host strains from the other source groups ($p < 0.008$). Phage numbers detected by enterococcal hosts isolated from wastewater and surface waters did not vary greatly from one another, but significantly higher numbers of phage were detected by host strains isolated from cattle and pig fecal runoff ($p < 0.008$).

Identification of Enterococcal Host Strains. Fourteen potential hosts demonstrating specificity to a single fecal source or source group were identified (Table 4). Human-specific host strains NRO8 and MW42 were both identified as being members of the species *E. faecalis*, and MW47 was identified as *Enterococcus faecium*. Pig-specific host strain (NRO24) was also identified as *E. faecium*. All cattle specific host strains were identified as *Enterococcus mundtii*, *Enterococcus casseliflavus*, or *Enterococcus gallinarum*. *E. casseliflavus* and *E. gallinarum* are commonly isolated from environmental samples, but it has been suggested that these species could be incorporated into the microbiota of the digestive tract following ingestion, and that their presence could therefore be related to diet.[27] The species classification of the host strains CR1 and NRO39 differed depending on which of the two identification methods was used. Host strains CR1 and NRO39 were identified as being members of the species *E. casseliflavus* and *E. gallinarum*, respectively, by API 20 Strep, whereas the simplified biochemical key of Manero and Blanch[25] identified the hosts as being members of the species *Enterococcus mundtii* and *E. faecium*, respectively. API 20 Strep failed to provide identification for three isolates, all of which were identified as *E. gallinarum* by the biochemical key.

DISCUSSION

The high specificity of phages infecting *Enterococcus* hosts (even down to a specific herd level) as witnessed in this study suggests that *Enterococcus* hosts have a potential role as MST tools. However, the lower level of sensitivity associated with high specificity represents a potential problem. Therefore, strains with lower specificity but with higher sensitivity (such as CR70) may prove more useful for MST applications than more specific strains (such as WFS7) in surface waters. Ideally a toolbox approach to the detection of human and nonhuman sources, utilizing several strains in parallel, may be advisible for future MST studies. These results also suggest that, when isolating potential enterococcal host strains for phage-lysis based MST applications, it is better to isolate agricultural hosts directly from liquid farmyard runoff, rather than from fresh pooled scats from individual animals. Isolation of enterococcal hosts directly from impacted surface waters also led to the discovery of strains useful for MST application, without negatively impacting host specificity. This may be due to the selection of more environmentally tolerant phages and enterococcal host strains.

The application of the tiered screening approach simplified and improved the efficiency of isolating new host strains and serves as a useful protocol for future isolation of phage hosts. At least one potential host strain was isolated for every sample analyzed (approximately one host strain for every 40 isolates), with the exception of the pooled cattle fecal sample. Although the geographical stability of the host strains isolated in this study was not determined, not all host strains used in phage-based MST techniques have shown geographical stability.[14] We therefore suggest that the approach presented here can be applied in other countries to isolate host strains suitable for particular regions. The cost of isolating and screening new *Enterococcus* host strains is considerably lower than that of developing molecular MST techniques, particularly those that are dependent on the construction of a library or database.[18] Culture-based phage MST techniques are relatively simple to carry out, do not require specialist expertise, and can be performed in laboratories equipped with basic microbiological apparatus.[17] In this study, the use of *Enterococcus* host strains also proved rapid with visualization of plaques possible within 4 h.

In conclusion, the findings of this study are significant in that they offer an insight into host-phage interactions, specificity, sensitivity, and suitability of phages infecting different *Enterococcus* strains for MST application. The high specificity of the enterococcal hosts isolated in this study demonstrates that phages infecting strains of *Enterococcus* spp. possess narrow host ranges similar to those previously reported for anaerobes such as *Bacteroides* spp. An effective protocol for the isolation of new enterococcal host strains suitable for MST application has been presented here which may be effectively used by others to isolate bacterial hosts for phage-lysis MST work in other parts of the world. Our relatively simple, rapid, and nonmolecular laboratory protocol (utilizing existing ISO phage methods), which is based on the use of a globally used fecal indicator bacterium, has considerable potential to support the identification of fecal contamination sources in both recreational waters and sources of raw drinking water. Our approach therefore has the potential to support Water Safety Plans and hence to help sustain global efforts to reduce the burden of waterborne disease in low-income countries.

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REFERENCES

(1) Craun, G. F.; Brunkard, J. M.; Yoder, J. S.; Roberts, V. A.; Carpenter, J.; Wade, T.; Calderon, R. L; Roberts, J. M.; Beach, M. J.; Roy, S. L. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. *Clin. Microbiol. Rev.* **2010**, *23* (3), 507–+.

(2) Le Guyader, F.; Haugarreau, L.; Miossec, L.; Dubois, E.; Pommepuy, M. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* **2000**, *66* (8), 3241–3248.

(3) Sinclair, R. G.; Jones, E. L.; Gerba, C. P. Viruses in recreational waterborne-disease outbreaks: A review. *J. Appl. Microbiol.* **2009**, *107* (6), 1769–1780.

(4) Craun, G. F.; Calderon, R. L.; Craun, M. F. Waterborne outbreaks caused by zoonotic pathogens in the USA. In *Waterborne Zoonoses: Identification, Causes and Control*; Cotruvo, J. A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D. O., Craun, G. F., Fayer, R., Gannon, V. P. G., Eds.; World Health Organization, IWA Publishing: London, 2004; pp 120−135.

(5) Soller, J. A.; Schoen, M. E.; Bartrand, T.; Ravenscroft, J. E.; Ashbolt, N. J. Estimated human health risks from exposure to

recreational waters impacted by human and non-human sources of faecal contamination. *Water Res.* **2010**, *44* (16), 4674–4691.

(6) United States Environmental Protection Agency (USEPA). Federal Water Pollution Control Act of 2002. As amended through P. L. 107−303. http://epw.senate.gov/water.pdf.

(7) Commission of the European Union (CEU), Directive 2000/60/EC of the European Parliament and of the Council of the 23 October 2000 establishing a framework for community action in the field of water policy. *Off. J. Eur. Communities* **2000**, *L327*, 1−72.

(8) Field, G. K.; Samadpour, M. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* **2007**, *41* (16), 3517–3538.

(9) Domingo, J. W. S.; Bambic, D; G.; Edge, T. A.; Wuertz, S. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. *Water Res.* **2007**, *41* (16), 3539–3552.

(10) Meays, C. L.; Broersma, K.; Nordin, R.; Mazumder, A. Source tracking fecal bacteria in water: A critical review of current methods. *J. Environ. Manage.* **2004**, *73* (1), 71–79.

(11) Jofre, J.; Bosch, A.; Lucena, F.; Girones, R.; Tartera, C. Evaluation of *Bacteroides fragilis* bacteriophages as indicators of the virological quality of water. *Water Sci. Technol.* **1986**, *18* (10), 167–173.

(12) Gourmelon, M.; Caprais, M. P.; Mieszkin, S.; Marti, R.; Wéry, N.; Jardé, E.; Derrien, M.; Jadas-Hécart, A.; Communal, P. Y.; Jaffrezic, A.; Pourcher, A. M. Development of microbial and chemical MST tools to identify the origin of the faecal pollution in bathing and shellfish harvesting waters in France. *Water Res.* **2010**, *44* (16), 4812–4824.

(13) Maite, M.; Payan, A.; Moce-Llivina, L.; Blanch, A. R.; Jofre, J. Differential persistence of of F-specific RNA phage subgroups hinders their use as single tracers for faecal source tracking in surface water. *Water Res.* **2009**, *43* (6), 1559–1564.

(14) Payan, A.; Ebdon, J.; Taylor, H.; Gantzer, C.; Ottoson, J.; Papageorgiou, G. T.; Blanch, A. R.; Lucena, F.; Jofre, J.; Muniesa, M. Method for isolation of *Bacteroides* bacteriophage host strains suitable for tracking sources of fecal pollution in water. *Appl. Environ. Microbiol.* **2005**, *71* (9), 5659–5662.

(15) Ebdon, J.; Maite, M.; Taylor, H. The application of a recently isolated strain of *Bacteroides* (GB-124) to identify human sources of faecal pollution in a temperate river catchment. *Water Res.* **2007**, *41* (16), 3683–3690.

(16) Vijayavel, K.; Fujioka, R.; Ebdon, J.; Taylor, H. Isolation and characterization of *Bacteroides* host strain HB-73 used to detect sewage specific phages in Hawaii. *Water Res.* **2010**, *44* (12), 3714–3724.

(17) Gómez-Doñate, M.; Payan, A.; Cortés, I.; Blanch, A. R.; Lucena, F.; Jofre, J.; Muniesa, M. Isolation of bacteriophage host strains of *Bacteroides* species suitable for tracking sources of animal faecal pollution in water. *Environ. Microbiol.* **2011**, *13* (6), 1622–1631.

(18) Jofre, J. Is the replication of somatic coliphages in water environments significant? *J. Appl. Microbiol.* **2009**, *106* (4), 1059–1069.

(19) Bonilla, N.; Santiago, T.; Marcos, P.; Urdaneta, M.; Domingo, J. S.; Toranzos, G. A. Enterophages, a group of phages infecting *Enterococcus faecalis*, and their potential as alternate indicators of human faecal contamination. *Water Sci. Technol.* **2010**, *61* (2), 293–300.

(20) Grabow, W.; Neubrech, T. E.; Holtzhausen, C. S.; Jofre, J. *Bacteroides fragilis* and *Escherichia coli* bacteriophages: Excretion by humans and animals. *Water Sci Technol.* **1995**, *31*, 223–230.

(21) Mendez, J.; Jofre, J.; Lucena, F.; Contreras, N.; Mooijman, K.; Araujo, R. Conservation of phage reference materials and water samples containing bacteriophages of enteric bacteria. *J. Virol. Methods* **2002**, *106* (2), 215–224.

(22) Anon. *ISO 7899-2: Water Quality. Detection and Enumeration of Intestinal Enterococci—Part 2: Membrane Filtration Method*; International Organisation for Standardisation: Geneva, Switzerland, 2000.

(23) Adams, M. H. *Bacteriophages*; Wiley Interscience: New York, 1959.

(24) Anon. *ISO 10705-2: Water Quality. Detection and Enumeration of Bacteriophages—Part 2: Enumeration of Somatic Coliphages*. International Organisation for Standardisation: Geneva, Switzerland, 2001.

(25) Manero, A.; Blanch, A. R. Identification of *Enterococcus* spp. with a biochemical key. *Appl. Environ. Microbiol.* **1999**, *65* (10), 4425–4430.

(26) Gawler, A. H.; Beecher, J. E.; Brandao, J.; Carroll, N. M.; Falcao, L.; Gourmelon, M.; Masterson, B.; Nunes, B.; Porter, J.; Rince, A.; Rodrigues, R.; Thorp, M.; Walters, J. M.; Meijer, W. G. Validation of host-specific bacteriodales 16S rRNA genes as markers to determine the origin of faecal pollution in Atlantic Rim countries of the European Union. *Water Res.* **2007**, *41* (16), 3780–3784.

(27) Layton, B. A.; Walters, S. P.; Lam, L. H.; Boehm, A. B. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *J. Appl. Microbiol.* **2010**, *109* (2), 539–547.

(28) Malakoff, D. Microbiologists on the trial of polluting bacteria. *Science* **2002**, *295* (5564), 2352–2353.