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Enterococcus phages as potential tool for identifying sewage inputs in the Great Lakes region

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ABSTRACT

Bacteriophages are viruses living in bacteria that can be used as a tool to detect fecal contamination in surface waters around the world. However, the lack of a universal host strain makes them unsuitable for tracking fecal sources. We evaluated the suitability of two newly isolated *Enterococcus* host strains (ENT-49 and ENT-55) capable for identifying sewage contamination in impacted waters by targeting phages specific to these hosts. Both host strains were isolated from wastewater samples and identified as *E. faecium* by 16S rRNA gene sequencing. Occurrence of *Enterococcus* phages was evaluated in sewage samples ($n = 15$) from five wastewater treatment plants and in fecal samples from twenty-two species of wild and domesticated animals (individual samples; $n = 22$). Levels of *Enterococcus* phages, F + coliphages, *Escherichia coli* and enterococci were examined from four rivers, four beaches, and three harbors. *Enterococcus* phages enumeration was at similar levels (Mean = 6.72 Log PFU/100 mL) to F + coliphages in all wastewater samples, but were absent from all non-human fecal sources tested. The phages infecting *Enterococcus* spp. and F + coliphages were not detected in the river samples (detection threshold < 10 PFU/100 mL), but were present in the beach and harbor samples (range = 1.83 to 2.86 Log PFU/100 mL). Slightly higher concentrations (range = 3.22 to 3.69 Log MPN/100 mL) of *E. coli* and enterococci when compared to F + coliphages and *Enterococcus* phages, were observed in the river, beach and harbor samples. Our findings suggest that the bacteriophages associated with these particular *Enterococcus* host strains offer potentially sensitive and human-source specific indicators of enteric pathogen risk.

Introduction

Concentrations of fecal indicator organisms (FIOs), such as *Escherichia coli* and enterococci, have been used to establish recreational water quality standards in the United States and throughout the world (Noble et al., 2010; Kinzelman et al., 2011); however, the relationship between human enteric pathogens and concentrations of FIOs are dependent on the source of these bacteria. Epidemiological studies have consistently shown that human sources of pathogens have higher disease risk to humans than non-human sources (Dufour, 1984; Wade et al., 2010). Current methods for enumerating FIOs from water samples may not distinguish between human and non-human sources; and therefore, the actual risk to human health cannot be accurately measured (Scott et al., 2002). This has led to the need for finding alternative FIOs that are consistently human specific (Stapleton et al., 2007; Stewart et al., 2013).

In the Great Lakes region, streams, rivers, and beaches routinely contain levels of FIOs exceeding recreational water quality standards. For instance, monitoring studies (Byappanahalli et al., 2006; Ishii et al., 2007; Gronewold et al., 2013) have detected high densities of FIOs in the beach environment that impact nearshore water quality, resulting in beach closures. However, the FIOs enumerated from the beach environment may not stem from human sources and thus do not represent actual human health risk. To gain a more accurate estimation of risks to recreational users, alternate FIOs that correctly identify human sewage contamination are needed. Bacteriophages (phages) infecting several groups of gut bacteria (e.g. *Bacteroides*, *E. coli, Salmonella, Enterococcus*) have been proposed as alternative FIOs because they better indicate the presence of human enteric pathogens, especially viruses, in contaminated waters (Allwood et al., 2003; Bonilla et al., 2010; Purnell et al., 2011). Advantages of using phages as a screening tool for fecal contamination include: 1) detection method does not involve the construction of time-consuming and expensive reference libraries (Ebdon et al., 2007), 2) phages are abundant in feces (Vijayavel et al., 2010), 3) phages are more resistant to sewage treatment processes and other environmental stressors than the bacteria they infect (Moce-Llivina et al., 2005), 4) phages have relatively narrow host ranges (Ebdon et al., 2012), 5) phages are more similar in size and morphology to human enteric viruses, thereby mimicking pathogenicity (Vijayavel et al., 2010),

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and 6) phages offer insight into the infectious state/viability of such viruses (Harwood et al., 2013).

While F + coliphages have been used as a reliable marker for fecal contamination worldwide (Luther and Fujioka, 2004; USEPA, 2001a, b), their human specificity (based on the detection of serotypes II and III) has been called into question due to the presence of these genogroups in certain non-human sources such as cattle, pig, horse and bird feces (Scott et al., 2002; Long and Sobsey, 2004). A more promising group appears to be phages capable of infecting certain strains of intestinal *Bacteroides* (e.g. GA-17, GB-124, ARABA-84) as these phages have been shown to be restricted to human feces (Ebdon et al., 2007; Payan et al., 2005). Moreover, phages infecting strict anaerobes are less likely to replicate outside the human gut due to their reliance on metabolically active hosts (Jofre, 2009). However, their enumeration methods require the culturing and handling of anaerobic hosts which is more technically demanding than the methods used for phages infecting aerobic (or facultative anaerobic) bacterial hosts.

Researchers have focused on other phage hosts that are relatively easier to work with because of the challenges with culturing anaerobic bacteria. Phages of *Enterococcus* spp. in particular have received significant attention in recent years. Studies have shown that phages infecting certain *Enterococcus faecalis* strains may be restricted to human sources (Bonilla et al., 2010; Santiago-Rodríguez et al., 2010, 2013). Phages infecting certain strains of other *Enterococcus* spp., such as *Enterococcus casseliflavus*, *Enterococcus mundtii*, and *Enterococcus gallinarum* were reported to be restricted to cattle and *Enterococcus faecium* presumably restricted to human and pig origins (Purnell et al., 2011). These findings are in broad agreement with earlier studies which showed that *E. faecalis* and *E. faecium* have consistently been identified as the dominant *Enterococcus* spp. in human feces (Chenoweth and Schaberg, 1990; Ruoff et al., 1990) and wastewaters (Manero et al., 2002). The use of *Enterococcus* hosts also is desirable since these bacteria are widely regarded as water quality 'indicators of choice' throughout the world.

Phages infecting *E. faecalis* and *E. faecium* hosts appear to have been consistently detected in sewage influents (Bonilla et al., 2010) which necessitates the identification of sewage specific fecal markers shedding at significantly higher levels than the majority of human enteric pathogens (i.e. a conservative risk). The objectives of the current study were to: 1) isolate new *Enterococcus* host strains from Michigan wastewaters in an attempt to determine whether the new hosts could yield higher levels of phages than previously reported methods; 2) evaluate occurrence of phages infecting *Enterococcus* hosts in wastewaters, 3) compare the concentrations of conventional FIOs (e.g. *E. coli,* enterococci and F + coliphages) with those of *Enterococcus* phages in wastewaters, and 4) assess the usefulness of *Enterococcus* phages for monitoring environmental waters in the Great Lakes region.

Materials and methods

Isolation of presumptive Enterococcus hosts

Presumptive *Enterococcus* strains were isolated from wastewater influent samples, collected from Michigan WWTPs on bile esculin agar (Livingston, 1978), using a method similar to the isolation of *Bacteroides* hosts (Payan et al., 2005). Briefly, dilutions of raw wastewater were plated onto the *Bacteroides* Bile Esculin (BBE) agar and incubated at 36 ± 2 °C for 44 ± 4 h. Bacterial colonies exhibiting a dark halo were plated onto duplicate BBE agar plates and incubated under both aerobic and anaerobic conditions (Payan et al., 2005), with some modifications: in the current research, instead of selecting strictly anaerobic, large opaque or glistening, transparent, raised colonies (typically 2–3 mm diameter), smaller, facultative anaerobic bacterial colonies (typically < 1 mm) were transferred to *Bacteroides* Phage Recovery Media (BPRM) or Tryptone Soy Broth (TSB) and incubated at 36 ± 2 °C for 18 ± 2 h, before being Gram stained. Gram-positive, presumptive *Enterococcus* isolates showing good overnight growth (under facultatively anaerobic conditions) were assigned sequential isolation numbers (e.g. ENT-1, 2, 3 etc.) prior to screening for host specificity, sensitivity and further confirmation. Only those isolates displaying the above qualities were selected for further testing in environmental waters.

Samples

Wastewater samples

Wastewater samples were collected in sterile 1 L polyethylene sampling bottles, kept in the dark at 4 °C and transported to Wayne State University for analysis. Wastewater influent samples were obtained for phage analysis from three Waste Water Treatment Plants (WWTPs) located in Michigan (cities of Ypsilanti, Detroit, and Monroe) and from two WWTPs located in Indiana (Michigan City and Chesterton, located in La Porte and Porter counties, respectively) collectively serving a population of around 3.5 million.

Non-human fecal samples

Individual fecal samples were also collected from 22 different wild and domesticated animals for phage analysis, including the Sandhill crane (*Grus canadensis*), herring gull (*Larus smithsonianus*), Canada goose (*Branta canadensis*), wild turkey (*Meleagris gallopavo*), chicken (*Gallus gallus domesticus*), mallard duck (*Anas platyrhynchos)*, mouse (*Mus musculus*), brown rat (*Rattus norvegicus*), Mongolian gerbil (*Meriones unguiculatus*), New Zealand white rabbit (*Oryctolagus cuniculus*), groundhog (*Marmota monax*), domestic dog (*Canis familiaris*), coyote (*Canis latrans*), red fox (*Vulpes vulpes*), domestic cat (*Felis catus*), bobcat (*Lynx rufus*), domestic pig (*Sus scrofa domesticus*), domestic sheep (*Ovis aries)*, domestic goat (*Capra aegagrus hircus*), horse (*Equus ferus caballus)*, domestic cattle (*Bos primigenius*), and white-tailed deer (*Odocoileus virginianus*), located at the Kensington Metro Park Animal Farm, Michigan; Howell Nature Center, Michigan, and the Division of Laboratory Animal Resources, Wayne State University, Michigan. In the laboratory, the fecal material was well mixed using a vortex mixer (WhirlimixerTM). One fecal sample from each animal was tested and thus 22 samples were evaluated; no waste lagoons were sampled in this study. For each sample, 1 g (wet weight) of fecal matter was suspended in 100 mL of phosphate buffered water (PBW; pH 6.8) and a decimal dilution series was performed before the samples were analyzed for the presence of phages.

Environmental samples

The final stage of the study involved testing environmental samples obtained during peak swimming season (Memorial Day to Labor Day). The samples were taken from surface waters on three different occasions from: 1) river sites, (Clinton River, Rogue River, Detroit River and Saginaw River), 2) recreational beaches (Metro Beach, Memorial Beach, Bay City State Recreational Area and Kensington Metro Beach Park), and 3) harbors used for boating and fishing activities, but not approved for bathing and swimming, and characterized by poor water circulation (Black Creek Yacht Club, Grosse Point Yacht Club, and Saginaw Bay Yacht Club). Each site was sampled once daily three times a week. All samples were collected in 1 L sterile polyethylene sampling bottles, kept in the dark and transported to Wayne State University and processed within 6 h of collection.

Enumeration of microorganisms

Bacterial indicators (E. coli and enterococci)

The levels of *E. coli* and enterococci were determined using the Colilert-18 and Enterolert reagents, respectively (IDEXX, Westbrook, Maine). Colilert-18 or Enterolert reagent was added to each of 100 mL of serially diluted (10^0, 10^{-1} and 10^{-2}) test water samples and mixed well. The individual mixture was poured into the corresponding QuantiTray-2000, (IDEXX, Westbrook, Maine). Trays were incubated at 37 °C for 18 h (*E. coli*) and at 41 °C for 24 h (enterococci). The wells