Simultaneous Detection of Feces-specific Bacteriophages of *Bacteroides fragilis* with a Duplex PCR Assay

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ABSTRACT

Bacteriophages of the *Bacteroides fragilis* strains HSP40 and RYC2056 are used as indicators of human-specific and general (non-host specific) fecal pollution in water bodies. However, conventional anaerobic cultivation methods require 1-2 days of incubation. To overcome this limitation, in this study, we developed a DNA-based method to simultaneously detect representative bacteriophages (B40-8 and B56-3) that infect *B. fragilis* strains HSP40 and RYC2056, respectively. Both phages yielded a 224-bp amplicon with the primer pair BT5414/BT5415, and an additional 152-bp PCR product was observed for B40-8 with the primer pair BT5579/BT5580. The detection limits for B40-8 and B56-3 were 10^{-5} and 10^{-4} ng of pure DNA, and 1 and 50 ng of DNA template when 5 and 5,000 PFU/mL were spiked into distilled water, respectively. The assay exhibited a higher sensitivity for sewage samples, with < 0.1 and 15 PFU/mL of phages infecting HSP40 and RYC2056, respectively. The assay did not produce false positive results for the *Bacteroides* phages PG76, HB13, and GA17 or for the enterococcal phages AIM06 and SR14. The assay also detected RYC2056 phages that were isolated from sewage samples and the phage B40-8 when it was spiked into raw sewage. Thus, the newly developed PCR assay demonstrated potential for the environmental monitoring of *Bacteroides* bacteriophages, decreasing the analysis time to a few hours.

1. INTRODUCTION

Many countries have fecal contamination problems for both inland and coastal bodies of water. Water that is contaminated with pathogens from human and animal feces poses varying levels of risk to swimmers, seafood and freshwater fish consumers, and individuals who directly or indirectly use water (Pandey et al., 2014; Soller et al., 2015; Soller et al., 2010). Groups of coliforms, including *Escherichia coli* and enterococcus, are the current biological indicators for monitoring water quality in most countries (European Union, 2006; National Environment Board, 1994; USEPA, 2012). However, the ability of fecal indicator bacteria (FIB) to multiply in water environments, particularly in tropical climates, can limit their use as fecal indicators (Fujioka et al., 2015). Interestingly, FIB have been shown to be unrelated to waterborne pathogenic microorganisms, increasing the need to discover better fecal indicators (Savichtcheva and Okabe, 2006). Additionally, to facilitate water quality restoration and water resource management, the identification of sources of pollution is essential. Several groups of intestinal and fecal micro-organisms have been shown to be specific to humans and animal hosts and can thus be used to indicate fecal sources. These microorganisms have been investigated in an area of research called 'microbial source tracking' (MST) (Sargent et al., 2011; Scott et al., 2002; Tran et al., 2015).

Bacteriophages that infect *Bacteroides*, an anaerobic genus of bacteria residing in homeothermic gastrointestinal tracts, are among the organisms studied for MST purposes (Blanch et al., 2006; Jofre et al., 2014; Leknoi et al., 2017; Payan et al., 2005; Savichtcheva and Okabe, 2006; Sirikanchana et al., 2014). Unlike FIB, the prevalence of *Bacteroides* phages has been demonstrated to be associated with

enteric viruses in the environment (Jofre et al., 2014; McMinn et al., 2017). *B. fragilis* strain HSP40 specifically identifies bacteriophages of human origin, whereas strain RYC2056 is used to detect phages from humans and animals; thus, these strains constitute human-specific and non-host specific fecal indicators, respectively (Gómez-Doñate et al., 2011; Jofre et al., 2014; Payan et al., 2005; Puig et al., 1999; Sirikanchana et al., 2014). The narrow bacterial host range of *B. fragilis* phages to a bacterial strain level is due to the presence of phage receptors on the cell wall, which can be inhibited by varying the amount of capsule present in different bacteria (Jofre et al., 2014; Klieve et al., 1991; Puig et al., 2001). The specific animal host association that benefits MST application might have been caused by differential phage-host co-evolution within animal hosts (Jofre et al., 2014). The traditional method for detecting *Bacteroides* phages is a plaque assay that uses a double-layer agar assay. However, the generation of strict anaerobic conditions, which are required for culturing the *Bacteroides* host and its phages, is a significant encumbrance (Araujo et al., 2001), limiting the utilization of this method in unequipped laboratories. Moreover, the culture method requires an 18- to 48-h incubation (Araujo et al., 2001; Sirikanchana et al., 2014). To decrease the analysis time, molecular techniques obviate the need to culture the bacterial host and phage. Polymerase chain reaction (PCR) equipment and reagents have become increasingly affordable to developing countries and provide results within a few hours (Ragheb and Jimenez, 2014; WHO, 2016). Molecular detection methods for B40-8 phages exist (Puig et al., 2000a; Puig et al., 2000b), but general fecal indicators are still needed. Phage B56-3 has been used as a representative phage of RYC2056 in many studies (Gómez-Doñate et al., 2011; Gomila et al., 2008; McLaughlin and Rose, 2006). In addition, high DNA homology has been previously demonstrated between the representative phage B40-8 and the other phages that infect *B. fragilis* strain HSP40 (Puig et al., 2000a). Therefore, in this study, we selected phages B40-8 and B56-3 as representative phages that infect *B. fragilis* strains HSP40 and RYC2056, respectively. The goal of this study was to establish a duplex PCR assay for the simultaneous detection of *B. fragilis* phages B40-8 and B56-3.

2. METHODOLOGY

2.1 Bacteria and bacteriophages

B. fragilis strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786), as well as their bacteriophages B40-8 (ATCC 51477-B1) and B56-3 (ATCC 700786-B1), were shipped from the American Type Culture Collection (ATCC, Manassas, VA, USA) in lyophilized form. The cultures were rehydrated and grown in *Bacteroides* Phage Recovery Medium (BPRM) (Araujo et al., 2001) and then subjected to anaerobic incubation as previously described (Sirikanchana et al., 2014). The bacterio-phages were propagated from single plaques and enumerated using the double-layer agar assay (Leknoi et al., 2017). Subsequently, phage suspensions were filtered twice with a 0.45-µm polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The filtrate was further concen-trated using Amicon Ultra 30K-15 Centrifugal Filter Units (Merck Millipore) following the manufacturer's instructions. The resulting phage stock was suspended in TM buffer and stored at 5°C. A spot test was also conducted to assess the ability of the phages to infect another bacterial host strain as previously described (Wangkahad et al., 2015).

2.2 DNA extraction

Bacteriophage and bacterial DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). DNA concentrations were measured by a NanoDrop 2000 UV-Vis Spectro-photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Prior to bacteriophage DNA extraction, any potentially contaminating bacterial host DNA in the bacteriophage stock was removed by following a previously described protocol with some modifications (Kot et al., 2014). Briefly, 200 µL of 1X DNase I buffer (Thermo Fisher Scientific) was added to the bacteriophage stock. The suspension was mixed thoroughly and incubated at 40°C for 30 min. Next, 5 U of DNase I enzyme was added, followed by gentle mixing. After incubating at 37°C for 30 min, 50 µL of 50 mM EDTA was added, followed by thorough mixing and DNA extraction. Confirmation of the absence of *Bacteroides* host DNA was conducted with PCR amplification of *B. fragilis* group 16S rRNA (Matsuki et al., 2002). DNA concen-trations were measured with a NanoDrop 2000 UV-Vis Spectrophotometer.

2.3 Primer design

Partial DNA sequences of the bacteriophages B40-8 and B56-3 were obtained with an Ion Torrent Personal Genome Machine™ System (Thermo Fisher Scientific), and de novo assembling of the sequencing data was performed using Velvet (Zerbino and Birney, 2008). Unique DNA regions were identified using the available *Bacteroides* phage B40-8 and B124-14 sequences (GenBank Accessions FJ008913.1 and HE608841.1) (Hawkins et al., 2008; Ogilvie et al., 2012) with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Specific primers for phages B40-8 and B56-3 were designed with Primer-BLAST (Ye et al., 2012).

2.4 Duplex PCR assay optimization

The designed duplex PCR primers were optimized for the PCR reaction and cycling conditions, especially for primer concentrations and annealing temperatures. A 50 µL PCR reaction contained 25 µL of 2x DreamTaq PCR Master Mix (Thermo Fisher Scientific), a 0.10 - 0.40 µM final concentration of each primer, varying amounts of DNA template (0.1 - 100 ng), and sterile distilled water. The reactions were run on a Mastercycler Pro thermocycler (Eppendorf, Hamburg, Germany). The PCR cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, an annealing step at temperatures ranging from 50 to 60°C for 30 sec, and an extension step at 72°C for 30 sec, with a final extension step at 72°C for 10 min. DNA products were electrophoresed in agarose gels and visualized using a Gel Doc XR system (BIO-RAD, Hercules, CA, USA). Positive controls using B40-8 and B56-3 template DNA, and no-template controls, were performed in each run.

2.5 Assay sensitivity

Varying amounts of B40-8 and B56-3 DNA, ranging from 10^{-7} to 10^{1} ng, were used as template in the duplex PCR assay. Detection limits were determined as the lowest amount of DNA template that resulted in visual bands and was confirmed by three replicates. In addition, B40-8 and B56-3 phage stocks, ranging from 5×10^{2} to 5×10^{5} PFU, were spiked into 100 mL of sterile distilled water. The suspensions were filtered with 0.22 µm mixed cellulose ester membranes (Merck Millipore). Then, the membrane filters were cut into small pieces, and the DNA was extracted from the cut filters using a ZR Fecal DNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The resulting DNA concentrations were measured with a NanoDrop 2000 UV-Vis Spectrophotometer before being used as DNA template for the duplex PCR assay. The detection limits, measured as PFU/mL, were determined as the lowest spiked phage concentrations that showed visual bands and were confirmed by three replicates.

2.6 Assay specificity

The specificity of the assays that were developed to specifically detect bacteriophages B40-8 and B56-3 was confirmed by testing the assays with bacteriophages that infect other *Bacteroides* strains. Bacteriophages that infect *B. fragilis* strain PG76, which was isolated from Spain, are reportedly present only in pig manure (Gómez-Doñate et al., 2011). Moreover, bacteriophages of *Bacteroides thetaiotaomicron* strains HB13 and GA17, isolated from municipal wastewater, appear to be specific to human fecal origin (Payan et al., 2005). These bacteria and their bacteriophages were kindly provided by Professor Juan Jofre from the University of Barcelona and were cultured in BPRM as previously described (Leknoi et al., 2017; Sirikanchana et al., 2014). In addition, bacteriophages of *Enterococcus faecalis* strains AIM06 and SR14, which have been observed to be prevalent in human sewage and polluted water in Thailand, were also used to evaluate the PCR assay for field applications in Thailand. These strains were cultured as previously described (Wangkahad et al., 2017).

2.7 DNA sequencing of PCR products

The expected DNA products that were visualized by gel electrophoresis were cut and extracted with a QIAquick Gel Extraction Kit (QIAGEN). The purified DNA products were cloned into *E. coli* DH5α competent cells using a pGEM-T Easy Vector System (Promega, Madison, WI, USA). Plasmids from the cloned *E. coli* isolates were extracted using a QIAprep Spin Miniprep kit (QIAGEN). The plasmid samples were sequenced using an automated DNA sequencer (Macrogen Inc., Republic of Korea). The resulting sequences were analyzed using BLAST (Altschul et al., 1990) against the nucleotide collection database.

2.8 Environmental sample testing

The ability of the duplex PCR assay to detect phages in raw sewage was assessed. Raw sewage samples were collected by grab sampling from influent sumps of hospitals that had more than 100 in-patient beds to represent the bacteriophage community as previously suggested (Blanch et al., 2006). Plaque forming units (PFU) of phages infecting *B. fragilis* strains HSP40 and RYC2056 were enumerated using a double-layer agar assay (Leknoi et al., 2017). One hundred milliliters of the water samples were filtered with 0.22-µm mixed cellulose ester membranes (Merck Millipore), and then subjected to DNA extraction using the ZR Fecal DNA MiniPrep kit (Zymo Research). The concentrations of the resulting DNA samples were measured with a NanoDrop 2000 UV-Vis spectro-photometer before being used as DNA template for the duplex PCR assay. Moreover, single plaques were isolated from sewage, cultured, and used to extract DNA, and the resulting DNA was used as the template to test whether the newly designed PCR assay could detect environmental phages. Additionally, B40-8 phages were spiked into raw sewage, and the duplex PCR assay was performed to determine the effect of the water matrix on the ability of the assay to detect bacteriophages.

3. RESULTS AND DISCUSSION

3.1 Duplex PCR assay development

A unique, 335-bp DNA region of phage B56-3 was retrieved (GenBank Accession MG372493; this study) that was not similar to any other microorganisms in the NCBI database, showing the specificity of the sequences (accession date: 15 October 2017). Another unique DNA fragment that was obtained from phage B40-8, which was 153-bp in length, was completely homologous to the 14790 - 14942-bp region of B40-8 GenBank accession FJ008913.1 (Hawkins et al., 2008) but did not match any other entries in the NCBI database. The formerly published nested primers for B40-8 also did not match this DNA region (Puig et al., 2000b). Duplex PCR primers were designed as shown in Table 1.

Table 1. Primers for the detection of bacteriophages B56-3 and B40-8

Primer	Sequence (5'→ 3')	Amplicon length (bp)	Bacteriophage
BT5414 (F)	ACC TCC TTG TCT GCG AGT TTG	224	B56-3, B40-8
BT5415 (R)	TTG TTA GCC GCA ACA GGG TG		
BT5579 (F)	GTT CCC CGT GCA TGA AAA G	152	B40-8
BT5580 (R)	TCA AGA ATA CAA GGA CCG GAC T		

The optimum duplex PCR reaction components and cycling conditions are shown in Tables 2 and 3. Phage B56-3 produced an amplicon that was 224-bp in length, while phage B40-8 produced 224- and 152-bp amplicons (Figure 1). The 224-bp amplicon from both phages contained similar sequences, but did not match B40-8 GenBank Accession FJ008913.1. The difference between our B40-8 sequence and the reference sequence (GenBank Accession FJ008913.1) was confirmed by a dissimilar restriction enzyme digestion pattern between our B40-8 isolate and that of the reference sequence (data not shown). The impurity of the B40-8 stock was unexpected, since the B40-8 stock culture was grown from a single plaque and no host DNA contamination was observed. Moreover, an inability of phage 56-3 to infect *B. fragilis* HSP40, as tested in this study, emphasized the low possibility of co-culturing of both phages.

Table 2. Optimized duplex PCR reaction components

PCR component	Vol (µL)
DNA template	varied
2x DreamTaq PCR Master Mix	25
10 µM BT5414 primer	1
10 µM BT5415 primer	1
10 µM BT5579 primer	1.5
10 µM BT5580 primer	1.5
Sterile distilled water	varied
Total	50

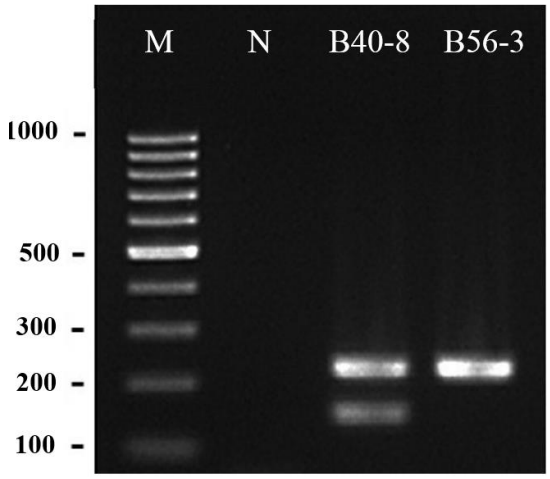

Figure 1. Duplex PCR. M, GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific); N, no-template control; B40-8 and B56-3 DNA templates.

Table 3. Duplex PCR cycling conditions

Step	Cycle	Temperature (°C)	Duration
Initial denaturation	1	95	5 min
Denaturation	35	95	30 sec
Annealing		52	30 sec
Extension		72	30 sec
Final extension	1	72	10 min
Hold		4	∞

3.2 Assay sensitivity and specificity

The sensitivity of the assay was demonstrated by the observed detection limits of 10^{-5} and 10^{-4} ng DNA for phages B40-8 and B56-3, respectively (Figures 2(a) and 2(b)). The lowest spiked concentration of 5 PFU/mL of phage B40-8 in distilled water provided a detection limit of 1 ng DNA per reaction (Figure 3(a)), comparable to that of previous reports for phage B40-8 and enteroviruses (Puig et al., 2000b; Puig et al., 1994). A higher detection limit of 50 ng was observed when phage B56-3 was spiked at 5,000 PFU/mL into distilled water (Figure 3(b)). Furthermore, although the specificity of the primers was observed when searching against the NCBI database, an investigation with DNA templates from other bacteriophages was also performed. The duplex PCR assay did not amplify DNA of *Bacteroides* phages PG76, HB13, and GA17, nor *E. faecalis* phages AIM06 and SR14, demonstrating the specificity of the assay. In addition, the assay could not amplify *B. fragilis* HSP40 and RYC2056 host DNA.

3.3 Environmental detection

The duplex PCR assay was used to detect bacteriophages from human sewage samples and was compared with the plaque assay (Table 4). The 224-bp amplicon was observed in sewage samples containing phages of RYC2056 at 15.5 and 233 PFU/mL at 100 ng total DNA template. The 152-bp amplicons were also positive, even in samples that yielded no PFU of HSP40 phages. Thus, the results indicated a higher assay sensitivity in sewage than in distilled water matrices. The dissimilar sensitivity of the PCR assay might be due to varying recovery percentages during the filtration and the DNA extraction processes. The membrane filtration method has been used to recover viruses and bacteriophages from water, but the recovery could be affected by different water matrices and phage types (Ahmed et al., 2015; Fuhrman et al., 2005; Harwood et al., 2013; McQuaig et al., 2009). The results also implied that PCR inhibition from environmental samples was less likely. The positive 152-bp PCR products in samples with negative PFU results for HSP40 phages also revealed a higher sensitivity of the PCR assay than the culture method, possibly because DNA-based detection methods can detect both infectious lytic bacteriophages, which can form plaques, and non-infectious phages (e.g., lysogenic phages or free

DNA). Unlike pathogenic microorganisms, for which it is important to differentiate an infectious form from non-infectious ones to accurately determine public health risk, detection of non-infectious bacteriophages is considered an advantage to increase the sensitivity of the assay to identify fecal sources.

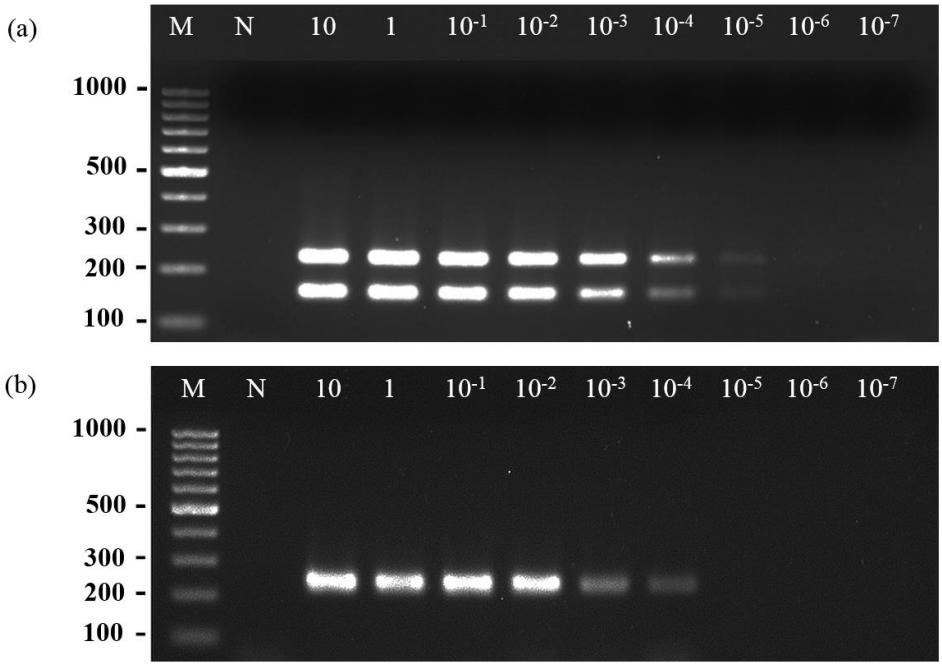

Figure 2. DNA detection limit with varying amounts of DNA template (ng) from B40-8 (a) and B56-3 (b). M, GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific); N, no-template control.

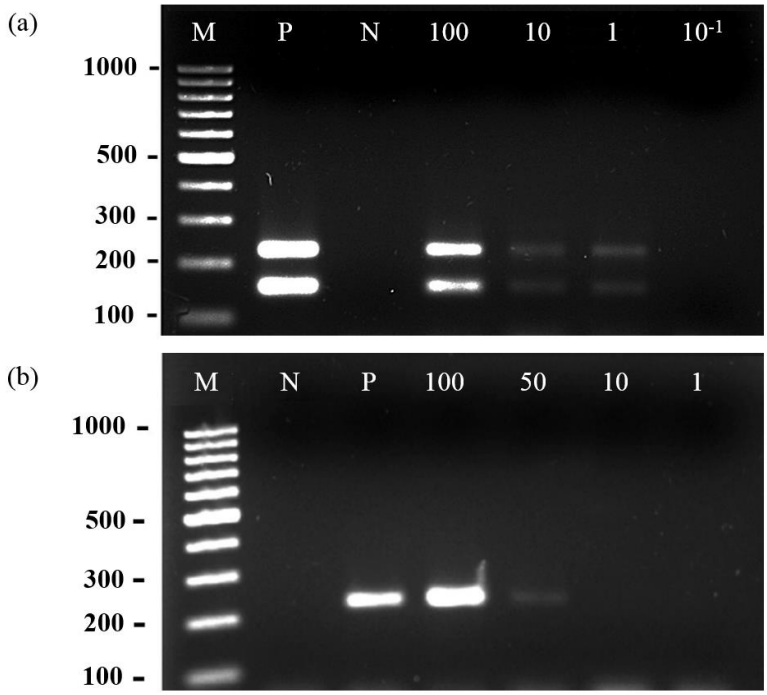

Figure 3. DNA detection limit of B40-8 with a spiked concentration of 5 PFU/mL (a) and of B56-3 with a spiked concentration of 5,000 PFU/mL (b) M, GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific); N, no-template control; P, positive control with 1 ng of DNA

Table 4. Duplex PCR assays and plaque assays of environmental samples

Environmental sample	Phages of *B. fragilis* HSP40 (PFU/mL)	Phages of *B. fragilis* RYC2056 (PFU/mL)	224-bp amplicon	152-bp amplicon
Sewage sample				
Sew 49	< 0.1	15.5	+[a]	+
Sew 51	< 0.1	1.3	-	+
Sew 53	< 0.1	233	+	-
Phage of RYC2056 isolated from sewage sample				
Sew 47	NA[b]	NA	+	-
Sew 48	NA	NA	+	-
Sew 49	NA	NA	+	-
Sew 51	NA	NA	+	-

[a] Positive amplicon was confirmed by DNA sequencing
[b] Not applicable

The PCR results from human sewage showed a higher sensitivity to phage B40-8 than B56-3, consistent with the results that were observed using distilled water and pure DNA template. The higher detection limits observed for B56-3 than those observed for B40-8 in both environmental and spiked distilled water samples would not limit field applications, since higher concentrations of phages that infect RYC2056 than those infecting HSP40 have been observed in geographically diverse field samples (Blanch et al., 2006; Muniesa et al., 2012; Sirikanchana et al., 2014). Furthermore, all phages that infect the bacterial host RYC2056 that were isolated from sewage samples were detected by the PCR assay, and all yielded the correct 224-bp amplicons, as confirmed by sequencing (Table 4). Because human sewage samples did not contain phages that infected HSP40 in the plaque assay, no such phage was isolated. Therefore, B40-8 phages were spiked at 4.2×10^5 PFU/mL into human sewage, and the results demonstrated that the PCR assay could produce both 224- and 152-bp amplicons with 10 ng of DNA template. These results, particularly in the context of the environmental samples assayed, underscore the potential of the duplex PCR assay developed in this study for environmental monitoring applications.

4. CONCLUSIONS

This study established the first duplex PCR assay for simultaneous detection of *Bacteroides* bacteriophages B40-8 and B56-3, which exhibited high sensitivity and specificity when tested with pure culture and environmental samples. The molecular-based assay could reduce the analysis time from the conventional culture methods, which require 1-2-day incubation times, down to a few hours, facilitating routine monitoring of environmental waters and outbreak events. In addition, the molecular information of *Bacteroides* phages could advance our knowledge of bacteriophage genomes, which is an essential component for further investigations of bacteriophage ecology, as well as the dynamics of virus-host interactions.

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