Occurrence of bacteriophages infecting *Aeromonas*, *Enterobacter*, and *Klebsiella* in water and association with contamination sources in Thailand

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ABSTRACT

The co-residence of bacteriophages and their bacterial hosts in humans, animals, and environmental sources directed the use of bacteriophages to track the origins of the pathogenic bacteria that can be found in contaminated water. The objective of this study was to enumerate bacteriophages of *Aeromonas caviae* (AecaKS148), *Enterobacter* sp. (EnspKS513), and *Klebsiella pneumoniae* (KlpnKS648) in water and evaluate their association with contamination sources (human vs. animals). Bacterial host strains were isolated from untreated wastewater in Bangkok, Thailand. A double-layer agar technique was used to detect bacteriophages. All three bacteriophages were detected in polluted canal samples, with likely contamination from human wastewater, whereas none was found in non-polluted river samples. AecaKS148 was found to be associated with human fecal sources, while EnspKS513 and KlpnKS648 seemed to be equally prevalent in both human and animal fecal sources. Both bacteriophages were also present in polluted canals that could receive contamination from other fecal sources or the environment. In conclusion, all three bacteriophages were successfully monitored in Bangkok, Thailand. This study provided an example of bacteriophages for potential use as source identifiers of pathogen contamination. The results from this study will assist in controlling sources of pathogen contamination, especially in developing countries.

Key words | bacteriophages, contamination sources, developing countries, pathogens, water quality

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INTRODUCTION

Microbiological water pollution has become a problem of increasing concern worldwide. Human and zoonotic waterborne pathogens can originate from both human and animal excreta, as well as from the environment, e.g., soil and sediments (Bagley 1985; Kuhn *et al*. 1997; Grimont & Grimont 2006; Cabral 2010). As feces is one of the main sources of a variety of pathogens, groups of microorganisms, i.e., fecal indicator bacteria, have long been used to indicate fecal contamination (Anonymous 2006; United States Environmental Protection Agency (USEPA) 2012). These non-pathogenic fecal indicators share the same origin as the fecal pathogens. In developed countries, mitigation measures have been implemented to control or reduce sources of fecal contamination, such as total maximum

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daily loads in the United States (USEPA 2013). As a result, the public health risk of pathogen exposure could eventually be reduced. However, for many developing countries, such corrective actions are rarely either available or strictly enforced. Therefore, the specific pathogenic agents should instead be regularly monitored and sources of contamination rapidly identified. This contrasts with the industrialized nations' emphasis on the need for developing countries to develop alternative general fecal indicators that directly track origins of pathogen contamination (Ahmed *et al.* 2010; Hagedorn *et al.* 2011; Harwood & Stoeckel 2011). Once the contamination sources are identified, subsequent remedial actions can be implemented.

Bacteriophages are viruses that infect bacterial cells. Some types of bacteriophage showed specificity to the human, rather than the animal, gut (Ogilvie *et al.* 2012). Bacteriophages have been reported to be able to predict the presence of pathogens of the same origin (Contreras-Coll *et al.* 2002). Bacteriophages of enteric bacterial hosts have been shown to be able to identify whether fecal pollution originated from humans or specific animal species as part of an expanding area called microbial source tracking (Payan *et al.* 2005; Field & Samadpour 2007; Kirs & Smith 2007; Santiago-Rodríguez *et al.* 2010). However, because not all waterborne pathogens are associated with fecal materials, depending on the pathogen of interest, the presence of the corresponding bacteriophage could also indicate environmental contamination sources, e.g., soil, vegetation, or natural water. Moreover, the culture technique used to enumerate bacteriophages is simple, straightforward, and economical, involving growing lawns of the bacteria of interest as a host (Araujo *et al.* 2001). Because phages specifically infect their host cells, observed plaques indicate that phages of the specific host genera/species/strains of interest are present in a tested water sample, with no additional confirmation steps required (Koskella & Meaden 2013). Therefore, bacteriophages appear to be a suitable tool to indicate the origins of their bacterial hosts.

It has been reported that the virulence levels of bacterial strains could be affected by geographic differences (Yu *et al.* 2007; Janda & Abbott 2010). Therefore, to provide direct information about risk of exposure to public health in specific regions, pathogens of local concern should be studied. The bacterial genera *Aeromonas*, *Enterobacter*, and *Klebsiella* have been reported as opportunistic bacterial pathogens for human and animals. In 2011, *Aeromonas caviae* was shown to be the agent that caused the highest rates of acute diarrhea in Thailand (Anonymous 2011). *Klebsiella pneumoniae* is the most common opportunistic pathogen that causes nosocomial infections in the patients of some hospitals in Thailand (Singthientrakool 2008). Furthermore, with the current widespread use of antibiotics, *Enterobacter* has recently been found more frequently as a pathogen (Grimont & Grimont 2006) and has been isolated from the chlorinated effluent of a municipal wastewater treatment plant (WWTP) in Mexico (Martinez-Hernandez *et al.* 2013) and from a hospital effluent in France (Barraud *et al.* 2013).

The ultimate goal of this research was to evaluate the potential use of bacteriophages of locally isolated pathogenic bacteria as contamination source identifiers. First, local pathogenic bacteria were isolated from human sewage samples. Species were identified together with their plaque appearance. Next, levels of the corresponding bacteriophages were enumerated in water sources with different levels of pollution, i.e., polluted urban canals and non-polluted rivers. The source specificity was examined by determining bacteriophage levels in domestic wastewater and in animal fecal materials. Finally, the relationship between microbial and physicochemical parameters from each source was assessed.

METHODS

Sample collection

For the isolation of bacterial host strains, human sewage samples were collected from the wastewater influent of a municipal wastewater treatment plant serving 120,000 persons, and of a hospital operating more than 80 beds. Samples for enumeration of bacteriophages, total coliforms (TCs), and *Escherichia coli* were collected from polluted urban canals (23 samples), non-polluted rivers (five samples), domestic wastewater (19 samples), and animal fecal samples (15 samples). Surface water samples were collected at the middle of the body of water 1 m below the surface. The

sites of the polluted canal samples were selected from community areas in Bangkok that had histories of high TCs, with likely contamination from human fecal material (Water Quality Management Office (WQMO) 2014). Non-polluted river samples were chosen from water sources that contained TC of less than 5,000 MPN 100 ml^{-1} (MPN: most probable number), considered as non-polluted water sources by the Thailand Surface Water Quality Standards (Pollution Control Department (PCD) 1994). Domestic wastewater samples were collected from facilities serving more than 100 individuals. All water samples were collected in 1-l decontaminated bottles with 3.33 ml of 3% $Na_2S_2O_3$ (sodium thiosulfate). For animal fecal samples, 10 g samples of animal feces, derived from a mixture of at least 20 individual animals, were collected from cattle and swine farms in Bangkok and nearby provinces. The transportation of water and animal samples on ice from the sites to the laboratory was completed within 3 h.

Enumeration of bacteriophages and indicator bacteria

Thirty-five milliliters of each water sample was centrifuged at 4,500 *g* for 45 min at 4 °C to separate the suspended solids from the clear liquid. The supernatant portion was filtered through a 0.22-μm polyvinylidene fluoride (PVDF) membrane (Millipore, Darmstadt, Germany), which has been reported not to retain bacteriophages (Tartera *et al.* 1992). The filtered liquid was subjected to bacteriophage determination in the subsequent step using a double-layer agar assay. The remaining pellet was resuspended in 6.4 ml of an eluent solution, and the pH was adjusted to 7.0 (Eaton *et al.* 2005c). The suspension was centrifuged at 4,500 *g* for 45 min at 4 °C. The pellet was discarded, and the supernatant was filtered through a 0.22-μm PVDF membrane. A double-layer agar technique was used to determine the number of phages in the filtered samples (Araujo *et al.* 2001). Briefly, 1–3 ml of the filtered sample was mixed with 1 ml of bacterial host culture grown to the log phase and 5–7 ml of semi-solid *Bacteroides* Phage Recovery Medium (BPRM). The mixture was poured onto a layer of solid BPRM agar. The plates were incubated at 37 °C for 24 h in aerobic conditions. The number of plaques was counted as plaque forming units (PFU) per 1 ml of water sample. For animal feces samples, 10 g of fresh feces was mixed with 35 ml of an eluent solution (Eaton *et al.* 2005c). Next, the pH was adjusted to 7.0, and the suspension was centrifuged at 4,500 *g* for 45 min at 4 °C. The pellet was discarded, the supernatant was filtered through a 0.22-μm PVDF membrane, and a double-layer agar assay was performed. Anaerobic incubation of the double-layer agar plates was performed in some experiments using a 3.5-l Anaerobic Jar (Oxoid, Hampshire, UK), which created anaerobic conditions with oxygen-eliminated sachets (Oxoid, Hampshire, UK). The bacteriophage detection limit ranged from 3 to 19 PFU 100 ml^{-1} in water samples and 2 PFU g $feces^{-1}$ in animal fecal samples. The detection limits varied among samples, depending on the volume of the water samples or the weight of the fecal sample being analyzed. In this study, percentages of data with values higher than the maximum detection limits, so-called threshold values, were reported instead of percentages of positive values. This method of reporting was followed to prevent giving misleading information: some positive values were obtained at lower than the highest detection limits.

TCs and *E. coli* were simultaneously enumerated using a membrane filtration technique with MI medium (USEPA 2002). In brief, serial dilutions were performed to obtain a suitable density of bacteria on the plates. Next, 10-ml water samples were filtered through 0.45-μm mixed cellulose filter membranes (Sartorius Stedim, Goettingen, Germany). Each membrane was placed onto a petri dish containing a layer of MI agar (BD, Franklin Lakes, New Jersey, USA). The plates were then incubated at 37 °C under aerobic conditions for 24 h. The number of bacterial colonies was counted as colony-forming units (CFU) per 100 ml of water sample. The detection limit for TC, and *E. coli* was 10 CFU 100 ml^{-1}.

Isolation and characterization of bacterial host strains

One milliliter of the water sample was spread onto *Bacteroides* Bile Esculin (BBE) agar and incubated under anaerobic conditions for 24 h at 37 °C (Payan *et al.* 2005). Each colony formed was streaked in duplicate onto BPRM agar plates (Araujo *et al.* 2001). One replicate plate was incubated under anaerobic conditions, while the other was incubated under aerobic conditions, for 24 h at 37 °C. Colonies that grew under both anaerobic and aerobic conditions showed facultative anaerobic characteristics. These isolates

were further investigated for their appearance using the Gram staining technique with microscopy. Presumptive bacteria of interest appeared as gram-negative, rod-shaped bacilli. Genera and species were further examined using traditional biochemical tests together with a commercial API® strip kit 20E (bioMérieux, Marcy l'Etoile, France). Confirmation of genera and species was performed using polymerase chain reaction (PCR) and by DNA sequencing of the 16S rRNA gene. Forward and reverse primers 5′ GAG-TTT-GAT-CCT-GGC-TC 3′ and 5′ GCT-ACC-TTG-TTA-CGA-CTT 3′ were used, respectively (Weisburg *et al.*1991). The 50 μl PCR mixture contained 10 μl 5× Phusion HF buffer, 1 μl 10 mM dNTPs, 2 μl each of forward and reverse primers, 50–250 ng template DNA, 1.5 μl dimethyl sulfoxide, 0.5 μl Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and sterile distilled water. The PCR cycling conditions comprised an initial denaturation at 98 °C for 30 s; 35 cycles of a denaturation step at 98 °C for 10 s, an annealing step at 42 °C for 30 s, and an extension step at 72 °C for 45 s; followed by a final extension step at 72 °C for 10 min. Single-band PCR products at lengths between 1,375 and 1,584 bp were observed on 1% agarose gel when processed by gel electrophoresis. The PCR products were sequenced by an automated DNA sequencer (Macrogen Inc., Seoul, the Republic of Korea). The DNA sequences were searched through the 'Basic Local Alignment Search Tool' (BLAST) against the National Center for Biotechnology Information databases for their similarities. All DNA sequences were submitted to the GenBank genetic sequence database (Bethesda, Maryland, USA), and the bacterial isolates were deposited in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

One bacterial host strain was isolated from an influent sump of a municipal WWTP, while the other two were isolated from a non-treated wastewater effluent from a hospital. The species confirmation of isolated bacteria is presented in Table 1. The bacterial strain KS148 belongs to *A. caviae*, while KS513 and KS648 belong to *Enterobacter* sp. and *K. pneumoniae* ssp. *pneumoniae*, respectively. These bacterial strains were used as hosts for subsequent bacteriophage detection. Because the isolated strains are members of facultatively anaerobic species of bacteria, aerobic vs. anaerobic incubating conditions were examined

Table 1 | Identification of bacterial host strains isolated from human sewage in Thailand

Bacterial strain	Isolation source	Total coliforms (CFU 100 ml^{-1})	*E. coli* (CFU 100 ml^{-1})	Biochemical tests	BLAST search					GenBank accession no.	Designated species	DSMZ accession no.
					DNA template length (bp)	% Coverage	% Max. identity	Most similar species				
KS148	Influent of a municipal wastewater treatment plant	3,230,000	3,180,000	*Aeromonas caviae*	1,360	100	100	*Aeromonas* sp. including *A. caviae*		KJ946377	*Aeromonas caviae*	DSM29415
KS513	Wastewater influent of hospital A	6,220,000	5,480,000	*Enterobacter* sp.	1,308	100	99	*Enterobacter* sp.		KJ946378	*Enterobacter* sp.	DSM29416
KS648	Wastewater influent of hospital A	6,220,000	5,480,000	*Klebsiella pneumoniae* ssp. *pneumoniae*	1,356	100	100	*Klebsiella pneumonia*		KJ946379	*Klebsiella pneumoniae* ssp. *pneumoniae*	DSM29417

to optimize the protocol for bacteriophage enumeration. Five replicates of each bacteriophage were assayed for plaque formation using the double-layer agar assay at appropriate dilutions and incubation under both aerobic and anaerobic conditions.

Isolation of bacteriophages and specificity to bacterial hosts

A plaque of bacteriophage in soft agar was cut carefully and transferred to a 1.5 ml microcentrifuge tube. One milliliter of BPRM broth was added to the tube. The plaque and BPRM broth were mixed by pulse-vortexing to yield a homogeneous solution. Then, 0.8 ml of homogeneous solution and 1 ml of the bacterial host culture were mixed with 5 ml of semi-solid BPRM agar. The mixture was poured onto the surface of BPRM agar and was allowed to solidify. The plate was then incubated for 24–28 h at 37 °C in aerobic conditions. Plaques of bacteriophages in soft agar were harvested again by scraping off the surface of agar plates. Then, the surface agar was vortexed and centrifuged at about 90 *g* for 25 min at 4 °C to settle the cellular debris and agar. The supernatant containing bacteriophages was filtered through a 0.22 μm pore size PVDF membrane. The filtrate, which contained bacteriophage suspension, was aliquoted and stored at 4 °C. Further preparation and purification of bacteriophages at high concentration were performed. Approximately 10^7 PFU of bacteriophages was added to 80 ml of BPRM broth with 8 ml of log-phase bacterial host. The culture was incubated at 37 °C under aerobic conditions for 18–24 h or until observed as a clear suspension. A control sample was also prepared similarly to above in a manner similar to that described above but without adding bacteriophage suspension. A control sample was used to indicate that lysed bacterial cells had occurred in the working suspension. Next, the suspension was transferred to 50-ml centrifuge tubes and centrifuged at 8,000 *g* for 10 min at 4 °C. The cell debris at the bottom of the centrifuge tubes was discarded. The supernatant containing bacteriophages was centrifuged for one additional time. Then, 250 μl of the mixture of 25% polyethylene glycol (PEG; M_r 8,000) and 2.5 mol l^{-1} sterile sodium chloride was added per 1.25 ml of the supernatant. The suspension was continuously stirred at 4 °C in a refrigerator for at least 30 min. Subsequently, the suspension was transferred to 50-ml centrifuge tubes and centrifuged at 8,000 *g* for 15 min at 4 °C. The supernatant was discarded. Then, 2–10 ml Tris-EDTA (TE) buffer (10:1 Tris:EDTA, pH 8.0) or buffered saline peptone water (8.50 g l^{-1} NaCl, and 1.00 g l^{-1} casein peptone) was added into the centrifuge tubes to resuspend the pellet of bacteriophage particles. The purified phage particles were stored at −20 °C until use.

Specificity of bacteriophages to bacterial hosts was conducted by spotting 5 μl of bacteriophage at approximately 10^2 PFU ml^{-1} onto a lawn of bacterial host. Bacterial hosts tested included the following: (1) *A. caviae* strain KS148, *Enterobacter* sp. strain KS513 and *K. pneumoniae* ssp. *pneumoniae* strain KS648 isolated in this study; (2) *Bacteroides fragilis* strain ATCC 51477, *B. fragilis* strain ATCC 700786, and *E. coli* strain ATCC 25922 ordered from American Type Culture Collection (Manassas, Virginia, USA); (3) *Bacteroides thetaiotaomicron* strain GA17 received from Professor Juan Jofre from University of Barcelona, Spain; and (4) two strains of enterococci bacteria isolated from wastewater in Bangkok by our laboratory. Bacteriophages AecaKS148, EnspKS513, and KlpnKS648 were isolated from wastewater as described above, while bacteriophages of *Bacteroides* bacteria were obtained together with their bacterial hosts. All *Bacteroides* strains and KS148, KS513, and KS648 were cultured in BPRM, whereas *E. coli* was grown in Luria-Bernati medium (BD, Franklin Lakes, New Jersey, USA) and enterococci in tryptic soy agar medium (BD, Franklin Lakes, New Jersey, USA). The plates were incubated at 37 °C for 24 h in aerobic conditions, except for *Bacteroides* species where anaerobic incubation was performed. Subsequently, the clear zone indicating cell lysis was observed.

Physicochemical analysis

On-site measurements of surface water samples, including pH, temperature, and dissolved oxygen (DO), were performed with YSI 60 and YSI Pro 2030 handheld instruments (YSI, Yellow Springs, Ohio, USA). Biochemical oxygen demand (BOD) and total suspended solids (TSS) were also measured with a modified azide method (Eaton *et al.* 2005b), and by drying at 103–105 °C (Eaton *et al.* 2005a), respectively.

Statistical data analysis

Analysis of data above and below the detection limits was performed (Helsel 2012). A non-normal distribution was observed using Q–Q plots by plotting the detected data with adjusted ranks from the non-detected data. Summary statistics were computed using the non-parametric Kaplan–Meier method with Efron bias correction. Significance of differences was conducted using the non-parametric generalized Wilcoxon score test unless stated otherwise. Correlation analysis was performed by the non-parametric Kendall's tau rank correlation method.

RESULTS

Characterization of isolated bacterial host strains and their bacteriophages

None of the bacterial strains showed significant differences in the number of plaques at aerobic vs. anaerobic incubation conditions ($p > 0.05$; Table 2). The characteristics of the plaques formed when the bacteriophages were incubated under aerobic and anaerobic conditions were also observed. Notably, gas bubbles were noticed on the upper layer of EnspKS513 and KlpnKS648 plates only under anaerobic conditions. These bubbles appeared almost similar to plaques and therefore interfered with the enumeration of plaques. Gas formation resulted from the ability of KS513 and KS648 bacterial strains to ferment glucose under anaerobic conditions, as determined by biochemical tests (data not shown). Overall, aerobic incubation conditions appeared to be more suitable for bacteriophage enumeration and were used in subsequent tests in this study. In addition, specificity of bacteriophages to bacterial hosts was conducted. None of bacteriophages AecaKS148, EnspKS513, and KlpnKS648 was able to infect other bacterial species, among the bacterial hosts tested. Also, the bacterial strains KS148, KS513, and KS648 did not allow infection of other bacteriophages.

Bacteriophage levels in water sources of different levels of pollution

A total of 23 urban canal samples with likely contamination from human fecal pollution were collected. Only 34.8% of AecaKS148 presented levels higher than the threshold value of 19 PFU 100 ml^{-1}, while 87.0% of both EnspKS513 and KlpnKS648 were detected above 19 PFU 100 ml^{-1} (Table 3; Supplemental Figure S1, available online at http://www.iwaponline.com/wh/013/204.pdf). Although the median concentration of KlpnKS648 was higher than that of EnspKS513, the plaque concentrations of the two strains were not significantly different, but the plaque formation of both was significantly higher than that of AecaKS148 (overall $p < 0.05$). River samples with TC and *E. coli* concentrations 2–3 orders of magnitude lower than the concentrations detected in canal samples were also tested (Table 3). None of AecaKS148, EnspKS513, or KlpnKS648 was detected at above 19 PFU 100 ml^{-1} in the river samples. Although the canal and river samples had dissimilar levels of TC and *E. coli*, the BOD did not appear to be much different (Table 4). Notably, the TSS was found to be slightly higher in the river samples than

Table 2 | Characteristics of bacteriophages under aerobic and anaerobic incubating conditions

	[a]Average no. of plaques (PFU)			Plaque appearance (incubating hours)	
Bacterial host	Aerobic condition	Anaerobic condition	*p*-value	Aerobic condition	Anaerobic condition
KS148	195	202	0.418	Round, clear plaque of 0.3–0.5 mm (24 h)	Round, clear plaque of 0.3–0.5 mm (24 h)
KS513	38	23	0.059	Round, clear plaque of 1.0–1.5 mm (20 h)	Round, clear plaque of 1.0–1.5 mm, and bubbles in plate (20 h)
KS648	179	147	0.418	Round, clear plaque of 1.5–2.0 mm (15 h)	Round, clear plaque of 1.5–2.0 mm, and bubbles in plate (15 h)

[a]Average no. from five replicates; *p*-value < 0.05 indicates significant difference (Wilcoxon signed-rank test).

Table 3 | Detected levels of total coliforms (TC), *E. coli*, AecaKS148, EnspKS513, and KlpnKS648 in surface water, domestic wastewater, and animal feces samples by incorporating non-detects using the non-parametric Kaplan–Meier test with Efron bias correction

	Summary statistics								
Parameter	**Percentage higher than the threshold value[a]**	**Minimum**	**Maximum**	**95th percentile**	**90th percentile**	**75th percentile**	**Median**	**25th percentile**	**10th percentile**
Canals ($n = 23$)									
TC (CFU 100 ml^{-1})	100.0	20,000	9,700,000	8,750,000	6,200,000	3,315,000	1,310,000	440,000	48,000
E. coli (CFU 100 ml^{-1})	100.0	8,500	2,700,000	2,700,000	2,450,000	1,495,000	435,000	135,000	10,000
AecaKS148 (PFU 100 ml^{-1})	34.8	N/A[b]	2,573	2,527	1,037	53	18	6	3
EnspKS513 (PFU 100 ml^{-1})	87.0	N/A	2,263	842	512	255	111	44	15
KlpnKS648 (PFU 100 ml^{-1})	87.0	N/A	1,131	1,091	941	341	209	119	19
Rivers ($n = 5$)									
TC (CFU 100 ml^{-1})	100.0	115	3,900	3,900	3,900	2,000	1,690	1,430	115
E. coli (CFU 100 ml^{-1})	100.0	55	1,235	1,235	1,235	1,215	500	450	55
AecaKS148 (PFU 100 ml^{-1})	0.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
EnspKS513 (PFU 100 ml^{-1})	0.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
KlpnKS648 (PFU 100 ml^{-1})	0.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Domestic wastewater ($n = 19$)									
TC (CFU 100 ml^{-1})	100.0	245,000	21,900,000	21,900,000	7,200,000	2,820,000	1,780,000	1,050,000	600,000
E. coli (CFU 100 ml^{-1})	100.0	125,000	10,600,000	10,600,000	6,320,000	2,440,000	820,000	470,000	310,000
AecaKS148 (PFU 100 ml^{-1})	73.7	N/A	5,805	5,805	4,333	2,417	1,292	N/A	N/A
EnspKS513 (PFU 100 ml^{-1})	73.7	N/A	497	497	471	380	121	7	4
KlpnKS648 (PFU 100 ml^{-1})	73.7	N/A	15,324	15,324	3,153	716	239	11	11
Hospitals ($n = 7$)									
TC (CFU 100 ml^{-1})	100.0	245,000	21,900,000	21,900,000	21,900,000	6,860,000	2,820,000	600,000	245,000
E. coli (CFU 100 ml^{-1})	100.0	125,000	10,600,000	10,600,000	10,600,000	6,320,000	2,440,000	330,000	125,000
AecaKS148 (PFU 100 ml^{-1})	42.9	N/A	1,292	N/A	1,292	44	N/A	N/A	N/A
EnspKS513 (PFU 100 ml^{-1})	71.4	N/A	380	380	380	350	55	7	7
KlpnKS648 (PFU 100 ml^{-1})	71.4	6	15,324	15,324	15,324	1,312	314	11	6
WWTPs ($n = 8$)									
TC (CFU 100 ml^{-1})	100.0	1,050,000	7,200,000	7,200,000	7,200,000	2,350,000	2,050,000	1,540,000	1,050,000
E. coli (CFU 100 ml^{-1})	100.0	310,000	5,300,000	5,300,000	5,300,000	1,410,000	820,000	750,000	310,000
AecaKS148 (PFU 100 ml^{-1})	100.0	29	5,805	5,805	5,805	4,333	3,780	1,733	29
EnspKS513 (PFU 100 ml^{-1})	87.5	4	497	497	497	425	318	189	4
KlpnKS648 (PFU 100 ml^{-1})	87.5	11	1,729	1,729	1,729	697	295	159	11
Residential buildings ($n = 4$)									
TC (CFU 100 ml^{-1})	100.0	690,000	2,130,000	2,130,000	2,130,000	2,130,000	1,090,000	790,000	690,000
E. coli (CFU 100 ml^{-1})	100.0	360,000	1,170,000	1,170,000	1,170,000	1,170,000	570,000	470,000	360,000
AecaKS148 (PFU 100 ml^{-1})	75.0	N/A	2,417	2,417	2,417	2,417	1,485	53	N/A
EnspKS513 (PFU 100 ml^{-1})	50.0	N/A	471	471	471	471	59	N/A	N/A
KlpnKS648 (PFU 100 ml^{-1})	50.0	N/A	3,153	3,153	3,153	3,153	224	N/A	N/A

(*continued*)

Table 3 | continued

Parameter	Summary statistics								
	Percentage higher than the threshold value[a]	Minimum	Maximum	95th percentile	90th percentile	75th percentile	Median	25th percentile	10th percentile
Mixed animal fecal samples ($n = 15$)									
TC (CFU g^{-1})	100.0	52,000	321,561,668	321,561,668	279,187,817	22,577,778	6,013,449	823,635	285,000
E. coli (CFU g^{-1})	100.0	49,000	253,416,149	253,416,149	201,353,638	19,022,222	4,380,403	680,394	265,000
AecaKS148 (PFU g^{-1})	0.0	N/A	N/A	N/A	2	N/A	N/A	N/A	N/A
EnspKS513 (PFU g^{-1})	46.7	N/A	1,756	1,756	274	48	2	N/A	N/A
KlpnKS648 (PFU g^{-1})	60.0	N/A	2,875	2,875	1,422	249	16	N/A	N/A
Mixed swine fecal samples ($n = 12$)									
TC (CFU g^{-1})	100.0	823,635	321,561,668	321,561,668	279,187,817	44,120,505	6,587,436	2,907,662	976,096
E. coli (CFU g^{-1})	100.0	680,394	253,416,149	253,416,149	201,353,638	38,678,328	5,332,198	2,907,662	876,494
AecaKS148 (PFU g^{-1})	0.0	N/A	N/A	N/A	2	N/A	N/A	N/A	N/A
EnspKS513 (PFU g^{-1})	58.3	N/A	1,756	1,756	274	181	12	N/A	N/A
KlpnKS648 (PFU g^{-1})	75.0	N/A	2,875	2,875	1,422	1,224	43	7	N/A

[a]The proportion of data higher than the maximum detection limit of 19 PFU 100 ml^{-1} for AecaKS148, EnspKS513, and KlpnKS648 in surface water and domestic wastewater, and of 2 PFU g^{-1} in animal feces samples.
[b]Not available.

in the canal samples. In the canal samples, many microbial parameter pairs presented statistically significant correlation ($p < 0.05$). EnspKS513 was shown to moderately correlate with TC and with *E. coli* ($\tau = 0.466$ and 0.478, respectively). KlpnKS648 slightly correlated with TC and with *E. coli* ($\tau = 0.312$ and 0.395, respectively). EnspKS513 also correlated with KlpnKS648 ($\tau = 0.387$). As expected, TC was correlated with *E. coli* ($\tau = 0.830$). Moreover, the physical parameters were observed to correlate with the microbial parameters in the canal samples. Temperature displayed an inverse correlation with TC, *E. coli*, and EnspKS513 ($\tau = -0.385$, -0.359, and -0.329, respectively). BOD was positively correlated with TC, *E. coli*, and KlpnKS648 ($\tau = 0.382$, 0.456, and 0.426, respectively). Lastly, pH demonstrated a correlation with KlpnKS648 ($\tau = 0.325$).

Bacteriophage detection in wastewater of human sources

Bacteriophage strains AecaKS148, EnspKS513, and KlpnKS648 were analyzed from domestic wastewater samples, which included human sewage from hospitals,

Table 4 | Physicochemical parameters and summary statistics of water samples collected from canals and rivers

Parameter	Canal				River			
	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD
pH	6.88	7.66	7.39	0.18	6.02	6.94	6.37	0.37
DO (mg l^{-1})	0.02	2.81	1.05	0.68	N/A[a]	N/A	N/A	N/A
Temperature (°C)	28.9	33.1	30.2	1.1	N/A	N/A	N/A	N/A
BOD (mg l^{-1})	17.0	68.0	37.9	13.7	30.5	37.0	34.8	2.4
TSS (mg l^{-1})	10.0	96.0	31.5	22.0	22.0	218.0	74.6	72.9

[a]Not available.

WWTPs, and residential buildings (Table 3). TC and *E. coli* were detected in all samples and were present in the range of 10^5–10^7 CFU 100 ml^{-1}. All three bacteriophage strains were found in most of the human wastewater samples, with 73.7% of data having values higher than 19 PFU 100 ml^{-1}. AecaKS148 showed the highest median concentration, with KlpnKS648 having the second-highest concentration and EnspKS513 having the lowest median concentration. A similar trend was observed in residential samples. However, no significant differences were observed among levels of AecaKS148, EnspKS513, and KlpnKS648 in all domestic wastewater samples, or in hospitals or residential building samples. When considering only WWTP samples, EnspKS513 and KlpnKS648 levels were not significantly different, but both were different from AecaKS148 levels (overall $p < 0.05$). No significant correlation was observed among microbial parameters when all domestic wastewater samples were considered, except for a correlation between TC and *E. coli* ($\tau = 0.813$).

When comparing the quality of water between canals and domestic wastewater samples, the TC of the canal samples was similar to that of domestic wastewaters, with no significant difference ($p > 0.05$), while *E. coli* concentrations were different between the two sample sources ($p = 0.03$). AecaKS148 levels were significantly different when present in domestic wastewaters compared to canal samples ($p = 0.006$), whereas levels of EnspKS513 and KlpnKS648 were not significantly different between two water sources ($p > 0.05$).

Bacteriophage detection in wastewater of animal sources

Animal fecal samples were collected from cattle and swine farms (Table 3). In swine samples, TC and *E. coli* were present in the range of 10^5–10^8 CFU g^{-1}. None of the AecaKS148 concentrations in the swine samples, or in any of the animal samples, was higher than 2 PFU g^{-1}, while 58.3–75.0% of EnspKS513 and KlpnKS648 was detected at concentrations higher than 2 PFU g^{-1}. Although the median concentrations of KlpnKS648 were higher than those of EnspKS513 in the swine samples and in all of the animal samples considered together, no significant differences were observed between the concentrations of these two strains ($p > 0.05$). Significant differences were found between these two strains and AecaKS148 (overall $p < 0.05$) in both types of samples. Notably, none of the three bacteriophage strains was positively detected in all three cattle samples with a detection limit of 2 PFU g^{-1}. TC and *E. coli* for cattle samples ranged from 5.2×10^4 to 5.3×10^5, and from 4.9×10^4 to 5.0×10^5 CFU g^{-1}, respectively, which were 1–4 orders of magnitude lower than the concentrations detected in the swine samples. Only TC and *E. coli* presented a significant correlation ($\tau = 0.962$) when considering all animal samples.

DISCUSSION

With the need for developing countries to control particular pathogens of concern in water pollution, bacteriophages of pathogens were evaluated for use as source identifiers. Notably, just as fecal indicators could indicate fecal contamination with no quantitative information on fecal pathogens, the amount of bacteriophages could not quantitatively imply concentrations of bacterial hosts in the water. Moreover, the presence of phages could imply risk of exposure to pathogens but could not guarantee the presence of bacterial hosts, and vice versa (Bigwood & Hudson 2009). This could be due to several factors, e.g., different growth and decay properties between phages and their bacterial hosts. However, unlike traditional fecal indicators that can replicate in the environment, the regrowth of bacteriophages in the water and sediment environment was probably not due to several factors, including limitations in the density and growth phase of bacterial hosts, as well as the presence of other bacteria and suspended solids (Wiggins & Alexander 1985; Grabow 2001; Muniesa & Jofre 2004).

This study enumerated all types of bacteriophages that could infect the bacterial host lawns, even though more than one type of phage might have been able to infect the same host strain. For example, *K. pneumoniae* strain B5055 was infected by five different types of bacteriophage (Kumari *et al.* 2010). Furthermore, in order to link the presence of bacteriophages to their bacterial hosts, the specificity properties of phages to their hosts need to be examined. This study demonstrated that all studied phages did not

cross-infect other bacterial species among the bacterial hosts tested. These infection profiles by bacteriophages could provide information about pathogen exposure in a way more specific to bacterial strains or more general to bacterial species/genera depending on the bacteriophages and bacterial hosts used. Previous research has shown that one type of bacteriophage can infect a variety of strains within similar species or a wide range of species within the same family. For example, bacteriophage Kpp95, isolated from *K. pneumoniae*, was able to infect many strains of *K. pneumoniae*, as well as strains of *Enterobacter agglomerans*, *Klebsiella oxytoca*, and *Serratia marcescens*, all of which belong to the family *Enterobacteriaceae* (Wu *et al.* 2007). Likewise, bacteriophage SFP10 was reported to infect both *Salmonella enterica* and *E. coli* O157:H7 (Park *et al.* 2012). Moreover, it should be noted that this study was performed simultaneously with an attempt to isolate *Bacteroides* species from human sewage samples. Therefore, the culture media used might not be the most appropriate for the bacterial groups *Aeromonas*, *Enterobacter*, and *Klebsiella.* BBE agar is a selective medium for *Bacteroides* but can allow the growth of bile-tolerant strains of other genera. The double-layer agar assays for bacteriophage enumeration were performed on BPRM. However, more economical and simple culture media could instead be used. Our results demonstrated that numbers of plaques formed on BPRM agar were not significantly lower than those on tryptic soy agar (data not shown).

This research focused on bacteriophages of *A. caviae* (AecaKS148), *Enterobacter* sp. (EnspKS513), and *K. pneumoniae* ssp. *pneumoniae* (KlpnKS648) in various sources of water and fecal samples, including polluted canals, non-polluted rivers, domestic wastewater, and animal feces. This study showed that AecaKS148 was found more frequently and at higher concentrations in domestic wastewater, especially in influents of municipal WWTPs, while it was rarely found in animal fecal materials. Also, significantly lower concentrations of AecaKS148 were detected in polluted canal samples. Consequently, AecaKS148, bacteriophages of *A. caviae*, could potentially be associated more with human sewage pollution. This is in agreement with a report that *A. caviae* was the dominant species among the *Aeromonas* group that were found in water with fecal pollution (Araujo *et al.* 1991). EnspKS513, bacteriophages of *Enterobacter* sp., and KlpnKS648, bacteriophages of *K. pneumoniae* ssp. *pneumoniae*, were detected in domestic wastewaters and in animal fecal samples, though the concentrations cannot be directly compared with those in water samples because the strains were analyzed on the basis of fecal weight. These bacteriophages were also found in canals, but not at significantly different levels than in domestic wastewaters. This result suggested that phage contamination in canals possibly came from more than one source.

Compared with AecaKS148, the bacteriophages EnspKS513 and KlpnKS648 showed lower concentrations in WWTPs but higher levels in animal samples and in canals. Therefore, EnspKS513 and KlpnKS648 could be more prevalent in various sources than AecaKS148. Higher levels of EnspKS513 and KlpnKS648, when compared with AecaKS148, in canal waters could have several implications. For instance, because the genera *Klebsiella* and *Enterobacter*, both belonging to the family *Enterobacteriaceae*, have also been found in water, sewage, and soil (Bagley 1985; Grimont & Grimont 2006; Cabral 2010), their bacteriophages could also come from the environment. Moreover, both strains might be more persistent in water environments or in water treatment processes of communities and buildings before disposal to canals. It was noticed that the viability of a purified stock of AecaKS148 when stored at −80 °C decreased more markedly than EnspKS513 and KlpnKS648 stocks when prepared at the same time and kept under similar storage conditions (data not shown). Preliminary tests also revealed that the viability of AecaKS148 decreased more rapidly than EnspKS513, but more slowly than KlpnKS648, when suspended in phosphate-buffered saline at pH 6.0 and 8.0 at both 4 °C and 30 °C (data not shown). Many factors, e.g., sunlight, salt concentration, particles, and dissolved organic matter, could also contribute to the survival of bacteriophages in natural water (Noble & Fuhrman 1997; Santiago-Rodriguez *et al.* 2010).

CONCLUSIONS

In conclusion, this bacteriophage detection method could qualitatively indicate origins of contamination, which are most likely to be the same sources of pathogenic bacterial

hosts. This study presented an instance of utilizing bacteriophages as source identifiers of pathogen contamination.

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