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Assessment of swine-specific bacteriophages of *Bacteroides fragilis* in swine farms with different antibiotic practices

Yuranan Leknoi, Skorn Mongkolsuk and Kwanrawee Sirikanchana

ABSTRACT

We assessed the occurrence and specificity of bacteriophages of *Bacteroides fragilis* in swine farms for their potential application in microbial source tracking. A local *B. fragilis* host strain, SP25 (DSM29413), was isolated from a pooled swine feces sample taken from a non-antibiotic farm. This strain was highly specific to swine fecal materials because it did not detect bacteriophages in any samples from human sewage, sheep, goats, cattle, dogs, and cats. The reference *B. fragilis* strain, RYC2056, could detect phages in swine samples but also detected phages in most human sewage and polluted urban canal samples. Phages of SP25 exist in the proximity of certain swine farms, regardless of their antibiotic use ($p > 0.05$). *B. fragilis* strain SP25 exhibited relatively high resistance to most of the veterinary antimicrobial agents tested. Interestingly, most farms that were positive for SP25 phages were also positive for RYC2056 phages. In conclusion, the swine-specific SP25 strain has the potential to indicate swine fecal contamination in certain bodies of water. Bacterial isolates with larger distributions are being studied and validated. This study highlights the importance of assessing the abundance of phages in local swine populations before determining their potential applicability for source tracking in local surface waters.

Key words | antibiotics, bacteriophages, *Bacteroides fragilis*, fecal pollution, microbial source tracking, swine

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INTRODUCTION

Animal husbandry is vital to the economies of many developed and developing countries. With the increasing number of domesticated swine, animal waste products have become a concern. Approximately 75% of emerging water pathogens may have animal origins (Cotruvo *et al.* 2004). In Thailand, in 2012, every day, fecal loads with a total biochemical oxygen demand (BOD) of 1.41×10^5 kg were released from swine farms into 25 river basins (Water Quality Management Bureau 2012). Human and swine fecal pollution sources must be differentiated, both to mitigate this pollution via controls implemented at the correct source and to estimate human health risks. Traditional fecal indicators, such as total coliforms, fecal coliforms, and *Escherichia coli*, cannot specifically assess fecal origin. In contrast, molecular methods of swine-specific microbial source tracking in swine excreta have been developed and validated, such as the detection of bacteria in the *Bacteroidales* order (Dick *et al.* 2005; Mieszkin *et al.* 2009; Heaney *et al.* 2015), eukaryotic mitochondrial DNA (Martellini *et al.* 2005), and porcine adenovirus types 3 and 5 (Wolf *et al.* 2010). However, for less-developed countries, culture techniques that are low-cost and require no highly skilled technicians are more suitable, especially for routine monitoring by public service laboratories. Bacteriophages have reportedly been associated with fecal pollution sources

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(Seurinck *et al.* 2005; Purnell *et al.* 2011; Jofre *et al.* 2014; Wangkahad *et al.* 2015). The detection of bacteriophages of *Bacteroides* and enterococci has been attempted, but 100% pig waste specificity has not yet been achieved (Gómez-Doñate *et al.* 2011; Purnell *et al.* 2011). In addition, differences in antibiotic practices must be studied for their effects on the prevalence of bacteriophages. This is due to variability in the gut microbial community that has been reported in pigs raised in the same geographical region but with different types of feed and antibiotic practices (Allen *et al.* 2011; Looft *et al.* 2012). In-feed antibiotics that serve as growth promoters and therapeutic agents are common in large-scale operations. These are used to maximize product yields. However, doses and withdrawal periods are controlled to prevent residue in pork meat. Conversely, to reduce costs, no antibiotics are given to pigs in some smallholder livestock systems.

The ultimate goal of this study was to evaluate the prevalence and specificity of bacteriophages detected by local *Bacteroides* host strains isolated from swine manure vs. foreign *Bacteroides* hosts for their potential application in microbial source tracking. First, local isolates of bacterial hosts were obtained, and their specificity in detecting phages from swine feces was assessed via testing against animal manure (sheep, goats, cattle, dogs, and cats), human sewage, and human-polluted canals. Second, surface waters with likely contamination from swine feces were examined to determine the usefulness of swine-specific bacterial hosts in detecting phages. Lastly, the prevalence of phages in swine populations was further investigated by assessing their geographical distribution and farming practice, i.e., farms that administer and do not administer antibiotics. The antimicrobial susceptibility of the swine-specific *Bacteroides* host strains was evaluated with common antibiotics, including chlortetracycline, colistin, lincomycin, sulfamethazine, tiamulin, and tylosin.

MATERIALS AND METHODS

Sample collection

Fresh fecal samples from various animals were obtained from animal farms and animal shelters in Central Thailand, including Pathum Thani, Phra Nakhon Si Ayuttaya, Nakhon Pathom, Suphanburi, Chachoengsao, and Ratchaburi. A minimum of 30 individual samples of similar origin, with 3.0 to 5.0 g of matter in each sample, were combined to create pooled fecal samples of swine (50), sheep (10), goats (9), cows (11), dogs (10), and cats (10). Fecal samples from swine that were not given antibiotics were collected from local farms that did not provide antibiotics to swine for disease prevention or growth-promoting purposes; in such facilities, antibiotics are only administered to sick animals for therapeutic reasons. Swine fecal samples containing antibiotics were obtained from industrialized farms that used antibiotics to improve growth rates and feed utilization, as well as to prevent and treat diseases. Two litres of human sewage were collected from the sewage effluents (no treatment) of hospitals (18) and municipal wastewater treatment plants (WWTPs; 3). Two litres of canal water samples were collected from polluted canals (20) in Bangkok that were located alongside heavily populated communities. Water samples were also taken from ponds (7) that receive water from nearby antibiotic-free swine farms. All surface water samples were collected 1 m below the surface. All samples were handled aseptically and transported on ice to the laboratory on the same day for analysis.

Bacteroides host strain isolation and species identification

Bacteroides host strains were isolated according to a previously published protocol, with slight modifications (Payan *et al.* 2005). In brief, 10 μl of a manure flushing water sample or 10 μl of eluent (Eaton *et al.* 2005c) (20 ml of eluent per 10 g of swine feces) were spread on *Bacteroides* Bile Esculin Agar (BBE; Livingston *et al.* 1978), and the plates were incubated at 35 °C for 24–48 h under anaerobic conditions. Only colonies with black or dark halos, which were considered presumptive isolates from within the *B. fragilis* group, were further tested for growth under both aerobic and anaerobic conditions. Isolates that grew only under anaerobic conditions were processed via Gram staining and microscopic analysis. Isolates that were Gram-negative and had a rod-shaped appearance were subsequently cultured in *Bacteroides* Phage Recovery Medium (BPRM) (Araujo *et al.* 2001). Cultures that showed good growth in broth were suspended in Bovine Serum Albumin (BSA)-sucrose (Araujo *et al.* 2001) and stored at −80 °C.

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These isolates were identified as *Bacteroides* species using biochemical tests and the API 20A anaerobe identification kit (bioMérieux, Marcy l'Etoile, France). Species identification was further confirmed via 16S rRNA gene sequencing. Bacterial DNA was extracted by boiling an isolate colony suspended in 20 μl of sterile distilled water for 10 min. Next, the suspension was centrifuged to remove cell debris, and the supernatant containing DNA was used as a DNA template in a polymerase chain reaction (PCR). The forward and reverse primers were 5′ GAG-TTT-GAT-CCT-GGC-TC 3′ and 5′ GCT-ACC-TTG-TTA-CGA-CTT 3′, respectively (Weisburg *et al.* 1991). Each 50-μl PCR mixture contained 10 μl of 5× Phusion HF buffer, 1 μl of 10 mM deoxynucleotide triphosphates (dNTPs), 0.5 μM each of the forward and reverse primers, 50–250 ng of template DNA, 1.5 μl of dimethylsulfoxide (DMSO), 0.5 μl of Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and sterile distilled water. The PCR cycling conditions consisted of 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, an extension step at 72°C for 45 s, and a final extension step at 72°C for 10 min. The reaction mixture was amplified using a Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR products were analyzed via agarose gel electrophoresis, and the DNA fragments were observed via the ultraviolet (UV) transillumination of an ethidium bromide-stained gel. Single DNA fragments with lengths between 1,375 and 1,585 bp were extracted with a QIAquick gel extraction kit (QIAGEN, Venlo, Netherlands) and sequenced using an automated DNA sequence analyzer (Macrogen Inc., Seoul, Republic of Korea). The resulting DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to determine their genomic identities. The DNA sequences were submitted to the GenBank genetic sequence database (Bethesda, MD, USA), and the bacterial isolates were deposited in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Bacteroides host strains RYC2056 and PG76 and their bacteriophages

B. fragilis strain PG76 was isolated from pig waste in Spain and was reported to be able to detect phages in all pig wastes, though cross-detection did occur with 50% of human sewage from Spain (Gómez-Doñate *et al.* 2011). *B. fragilis* strain RYC2056 (ATCC 700786), isolated from a hospital in Spain, was reported to be a non-host-specific fecal indicator because it was found in both human and animal feces in European and Mediterranean areas (Payan *et al.* 2005; Jofre *et al.* 2014). It has been detected in human wastewaters and swine manure in Thailand (Sirikanchana *et al.* 2014). Hence, these two bacterial host strains were selected as references for use in assessing the new bacterial host. *B. fragilis* strain RYC2056 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) in freeze-dried form. Upon arrival, the lyophilized bacterial sample in the vial was rehydrated with 10 ml of BPRM broth and incubated under anaerobic conditions at 35°C for 18 ± 2 h. *B. fragilis* strain PG76 was kindly provided by Professor Juan Jofre, University of Barcelona, Spain. All bacterial strains were stored at −80°C after suspension in BSA-sucrose (Araujo *et al.* 2001).

Bacteriophage detection in wastewater and fecal samples

Thirty-five millilitres of a water sample were centrifuged at 1,250 × g for 20 min at 4°C. The supernatant was filtered through a polyvinylidene fluoride (PVDF) membrane (Millipore, Darmstadt, Germany) to remove any contaminating bacteria (Tartera *et al.* 1992), followed by a double-layer agar assay. Bacteriophages were further eluted from the sediment solids, following a published protocol (Eaton *et al.* 2005c). In brief, 6.4 ml of eluent with a pH of 7.0 were added to the pellet taken from the water sample, and these were mixed together vigorously. For the pooled animal fecal samples, approximately 10 to 15 g of feces were mixed with 45 to 60 ml of an eluent solution before adjusting the pH to 7.0. Next, the mixing speed was reduced, and mixing was continued for 30 min while the pH was readjusted to 7.0. The suspension was further centrifuged at 1,250 × g for 20 min at 4°C. The supernatant was filtered through a PVDF membrane prior to double-layer agar assay, and the remaining pellet was discarded. The double-layer agar method was used for phage enumeration (adapted from Araujo *et al.* 2001). Briefly, frozen bacterial aliquots were grown in BPRM broth and incubated under anaerobic

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conditions at 35°C for 20–24 h, until the bacterial concentration reached approximately 10^8 colony forming units (CFU) ml^{-1} in the late log phase. The growth curves of each batch were charted to identify the proper incubation time. Then, 1–3 ml of the bacteriophage sample and 1 ml of the *Bacteroides* culture were mixed with 5–7 ml of semi-solid BPRM agar, and the mixtures were poured onto the surfaces of BPRM agar plates. The semi-solid agar was allowed to solidify, and the plates were incubated for 18 ± 2 h at 35°C in an anaerobic jar with an AnaeroGen sachet (Oxoid, UK) to create anaerobic conditions. The phages were counted by considering plaque forming units (PFU).

Measurement of microbial and physicochemical parameters

Total coliform and *E. coli* measurements were conducted via the membrane filtration method, using MI agar (United States Environmental Protection Agency (USEPA) 2002). Briefly, water samples were serially diluted with phosphate buffer solution. Appropriate dilutions were filtered through a mixed cellulose ester membrane with a 0.45-μm pore size (Sartorius, Goettingen, Germany) and then plated on MI agar (BD, Franklin Lakes, USA) supplemented with cefsulodin (Sigma Aldrich, St Louis, USA). The plates were incubated at 35°C for 24 h, and colonies were counted under natural light and 366-nm UV light. pH, BOD, and total suspended solids were measured using the electrometric method, the modified azide method (Eaton *et al.* 2005b), and drying at 103–105°C (Eaton *et al.* 2005a), respectively.

Antibiotic susceptibility testing of *Bacteroides* host strains SP25 and RYC2056

The antimicrobial susceptibility testing of *B. fragilis* strains SP25 and RYC2056 was performed using a broth microdilution method for anaerobic bacteria (Clinical and Laboratory Standards Institute 2012). Chlortetracycline hydrochloride, colistin sulfate salt, lincomycin hydrochloride, sulfamethazine, tiamulin hydrogen fumarate, and tylosin phosphate (Sigma Aldrich) were purchased for the test. The minimal inhibitory concentration (MIC) endpoint was set as the lowest concentration of the antibiotic that prevented growth. Antibiotic concentrations tested ranged from 0.25–1,024 μg ml^{-1}. Each antibiotic test was performed in triplicate for each bacterial strain.

Statistical analysis

Because the experimental data contained multiple detection limits, to prevent bias, the results were presented as the number of samples that showed higher concentrations than the highest detection limit, rather than the number of positive samples. Moreover, environmental detection data were calculated by incorporating data that were lower than the detection limits (Helsel 2012). A Q-Q plot revealed that all datasets were not normally distributed. Consequently, nonparametric statistics were calculated. Descriptive statistics were computed using the Kaplan-Meier method with Efron bias correction. Significant differences were determined using the generalized Wilcoxon test. The paired Prentice Wilcoxon test was executed as a nonparametric test for paired differences. Correlation analysis was performed using the nonparametric Kendall's tau method, where tau-a (τ_a) is a nonparametric correlation coefficient, to acknowledge all tie ranks suitable for non-detected data with multiple detection limits (Helsel 2012; Sirikanchana *et al.* 2014; Wangkahad *et al.* 2015).

RESULTS

Bacteroides host strain isolation and species identification

Ninety-one local *Bacteroides* strains were isolated from swine fecal samples collected from farms that did not administer antibiotics (Table 1). The isolated strains were screened based on their strictly anaerobic, Gram-negative, and rod-shaped characteristics, and during the final step, presumptive *Bacteroides* strains were tested for detectable bacteriophages. Of 21 presumptive *Bacteroides* isolates, only strain SP25 could detect phages in a set of three swine fecal samples from non-antibiotic farms. Strain SP25 was further characterized (Table 2), and both biochemical tests and sequence analysis confirmed the isolate as *B. fragilis*. This bacterial host strain was subsequently tested for its efficacy in detecting phages specific to swine feces.

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Table 1 | Isolation of *Bacteroides* host strains from swine feces

Source	[a]Step 1	[b]Step 2	[c]Step 3	[d]Step 4	[e]Step 5
Non-antibiotic swine farms	91	56	35	21	SP25

[a]Step 1 = number of colonies that showed dark halos on BBE agar plate.
[b]Step 2 = number of isolates that grew only under anaerobic conditions, not under aerobic conditions.
[c]Step 3 = number of isolates that showed Gram-negative and rod-shaped characteristics.
[d]Step 4 = number of isolates that grew well in BPRM broth.
[e]Step 5 = isolates that could detect phages in another three swine fecal samples from non-antibiotic farms.

Table 2 | Characteristics of *Bacteroides* strain SP25

Characteristics	Description
Colony appearance	White, round
Plaque appearance	Translucent, 1–2 mm in size (24 h incubation)
Biochemical test	*Bacteroides fragilis*
DNA template length (bp)	1,331
BLAST search	
% Coverage	100
% Max identity	100
Most similar species	*Bacteroides fragilis*
Accession date	Jan 12, 2016
GenBank accession no.	KM502963
DSMZ accession no.	DSM29413

Table 3 | Summary statistics of bacteriophages detected by *B. fragilis* strains PG76, RYC2056, and SP25 in pooled swine fecal samples as calculated by incorporating data below detection limits

Description	Phages of PG76	Phages of RYC2056	Phages of SP25
Antibiotic farms			
Total no. of pooled samples	[a] –	24	24
No. of samples higher than [b]HDL	–	11	8
Detection limits (PFU g^{-1})	–	0.65–2.6	0.65–2.6
Max (PFU g^{-1})	–	6,173.2	869.6
Median (PFU g^{-1})	–	–	1.0
Mean (PFU g^{-1})	–	492.6	49.3
S.E. (PFU g^{-1})	–	282.8	37.5
Non-antibiotic farms			
Total no. of pooled samples	17	26	26
No. of samples higher than HDL	0	12	7
Detection limits (PFU g^{-1})	3.2	0.89–3.2	0.89–3.2
Max (PFU g^{-1})	<3.2	3,979.2	581.3
Median (PFU g^{-1})	<3.2	2.6	1.0
Mean (PFU g^{-1})	<3.2	455.6	50.9
S.E. (PFU g^{-1})	[c]NA	206.9	30.5

[a]Not measured.
[b]Highest detection limit.
[c]Not available.

Prevalence of bacteriophages of *B. fragilis* strains PG76, RYC2056, and SP25 in pooled swine fecal samples

B. fragilis strain SP25 was compared with the currently available *B. fragilis* strains PG76 and RYC2056 in terms of their phage detection ability for pooled swine fecal samples in Thailand (Table 3). None of the samples from the 17 farms not administering antibiotics was positive for phages of PG76, with a detection limit of 3.2 PFU g feces^{-1}. Consequently, strain PG76 was considered unsuitable for detecting phages specific to pigs in Thailand and was not tested further.

Seventeen out of fifty swine fecal samples showed positive detection of strain SP25 phages in a range of 1.0–869.6 PFU g feces^{-1}, with sample detection limits ranging from 0.65 to 3.2 PFU g feces^{-1} (Table 3). Of the 17 SP25-phage-positive samples, 15 were higher than the highest detection limit of 3.2 PFU g feces^{-1}. The median and 75th percentile for SP25 phages in swine fecal samples were 1.0 and 9.8 PFU g feces^{-1}, respectively. In comparison, strain RYC2056 phages were found in 26 out of 50 pooled swine fecal samples; 23 of these were detected at levels higher than the highest detection limit of 3.2 PFU g feces^{-1} (Table 3), and the other three positive samples were detected at 1.0, 2.5, and 2.6 PFU g feces^{-1}. Strain RYC2056 phages were detected in a range of 1.0 to 6,173.2 PFU g feces^{-1}, with the median and 75th percentile being 2.6 and 259.0 PFU g feces^{-1}, respectively. The phages of strains SP25 and RYC2056 were simultaneously present in 14 pooled swine fecal samples at concentrations higher than 3.2 PFU g feces^{-1} (Figure 1). Interestingly, most of the positive samples (16 out of 17 samples) for SP25 phages were co-detected with RYC2056 phages. Therefore, when samples

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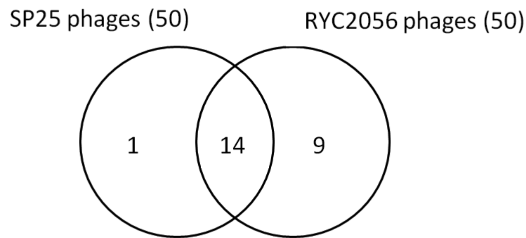

Figure 1 | Diagram showing numbers of pooled samples that presented detectable phages of *B. fragilis* strains SP25 and RYC2056 (higher than 3.2 PFU g^{-1}).

were screened as positive for RYC2056 phages, 61.5% (16 out of 26 samples) of them were positive for SP25 phages. Significantly lower concentrations of SP25 phages were detected in the pooled swine fecal samples than phages of strain RYC2056 ($p = 0.000$, paired Prentice Wilcoxon test). Furthermore, in pig fecal samples, a moderate correlation between both phages was noted (tau-a 0.329; Supplemental Table S1, available with the online version of this paper). The abundance of fecal indicator bacteria, i.e., total coliforms and *E. coli*, in pig feces was analyzed to determine its relationship with phage abundance, but no significant correlation was observed (Supplemental Table S1).

Specificity assessment of *B. fragilis* strains RYC2056 and SP25 in pooled animal fecal samples

Both *B. fragilis* strains RYC2056 and SP25 were evaluated for their specificity to swine fecal samples by testing against pooled fecal samples of other animals, including sheep, goat, cow, dog, and cat feces. None of the other animal fecal samples were positive for phages of both strains (Table 4). Therefore, bacteriophages of both strains SP25 and RYC2056 appeared to be specific to swine fecal materials, with no cross-detection with the feces of the other animals tested.

Specificity assessment of *B. fragilis* strains RYC2056 and SP25 in human sewage and surface water

Human sewage and surface water samples were collected and tested for phages infecting *B. fragilis* strains RYC2056

Table 4 | Summary statistics of bacteriophages detected by *B. fragilis* strains SP25 and RYC2056 in pooled animal fecal samples and water samples, as calculated by incorporating data below detection limits

Description	Sheep	Goats	Cows	Dogs	Cats	Human sewage	Urban canals	Ponds near swine farms
Phages of RYC2056								
Total no. of pooled samples	10	9	11	10	10	21	20	7
No. of samples higher than [a]HDL	0	0	0	0	0	20	19	2
Detection limits (PFU g^{-1} or PFU 100 ml^{-1})	0.65–1.3	1.2–1.3	0.65–1.5	1.1–1.3	1.2–1.3	19.1	19.1	16.7–19.0
Max (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	5,699.0	4,543.0	153.3
Median (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	742.9	447.0	8.0
Mean (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	1,290.9	909.0	30.4
S.E. (PFU g^{-1} or PFU 100 ml^{-1})	NA	NA	NA	NA	NA	352.3	279.1	23.3
Phages of SP25								
Total no. of pooled samples	10	9	11	10	10	21	20	7
No. of samples higher than [a]HDL	0	0	0	0	0	0	0	2
Detection limits (PFU g^{-1} or PFU 100 ml^{-1})	0.65–1.3	1.2–1.3	0.65–1.5	1.1–1.3	1.2–1.3	19.1	19.1	16.7–19.0
Max (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	<19.1	<19.1	61.7
Median (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	<19.1	<19.1	NA
Mean (PFU g^{-1} or PFU 100 ml^{-1})	<1.3	<1.3	<1.5	<1.3	<1.3	<19.1	<19.1	29.5
S.E. (PFU g^{-1} or PFU 100 ml^{-1})	NA	NA	NA	NA	NA	NA	NA	8.4

[a]Highest detection limit.

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and SP25 (Table 4). Phages from strain SP25 were not found in any of the 21 human sewage samples or 20 human-polluted urban canals. In contrast, strain-RYC2056 phages were present in almost all human sewage samples and polluted canal samples tested. The 25th, 50th, and 75th percentiles and maximum concentrations of strain-RYC2056 phages in human sewage samples were 338.3, 742.9, 1,472.0, and 5,699.0 PFU 100 ml^{-1}, while those in urban canal samples were 131.0, 447.0, 1,445.0, and 4,543.0 PFU 100 ml^{-1}, respectively. To evaluate the applicability of phage detection in surface water with potential swine fecal contamination, seven ponds near non-antibiotic swine farms were tested. SP25 phages were found in two pond samples. RYC2056 phages were detected in three pond samples, two of which were higher than the highest detection limit of 19.0 PFU 100 ml^{-1}. SP25 phages were not detected in the pond samples that were positive for strain RYC2056 phages and vice versa. This indicated that RYC2056 could detect human-polluted samples, while SP25 was very specific to swine fecal pollution. In an attempt to better understand the presence and prevalence of phages, the microbial and physicochemical parameters were measured, and their values are presented in Supplemental Table S2 (available with the online version of this paper). No significant correlation between phages and other parameters were noticed, except in urban canal samples, where RYC2056 phages showed a significant correlation with total coliforms (tau-a 0.663; Supplemental Table S1).

Investigating the effects of in-feed antibiotics on the detection of bacteriophages in swine feces and the *in vitro* susceptibility of bacterial hosts

To examine whether antibiotics used on farms had an effect on the prevalence and concentrations of bacteriophages in swine fecal samples, phages of SP25 and RYC2056 were analyzed based on pooled swine fecal samples from farms that used or did not use antibiotics (Table 3). SP25 phages were detected in 9 of 24 pooled swine fecal samples from farms using antibiotics, one of which presented a concentration lower than the highest detection limit of 2.6 PFU g $feces^{-1}$. The 50th and 75th percentiles and maximum concentrations of SP25 phages in swine fecal samples that used antibiotics were 1.0, 9.8, and 869.6 PFU g $feces^{-1}$, respectively. Among swine farms not using antibiotics, 8 of 26 were positive for phages of SP25, 7 of which were higher than the highest detection limit of 3.2 PFU g $feces^{-1}$. The 25th, 50th, and 75th percentiles and maximum concentrations were 1.0, 1.0, 15.0, and 581.3 PFU g $feces^{-1}$, respectively. There were no significant differences in the number of strain-SP25 phages for both types of farms ($p > 0.05$, generalized Wilcoxon test). RYC2056 phages were detected in 11 of 24 swine fecal samples from farms using antibiotics and in 15 of 26 samples from farms without antibiotic use. The 75th percentile and maximum concentrations of strain RYC2056 phages in swine fecal samples from farms that used antibiotics were 318.8 and 6,173.2 PFU g $feces^{-1}$, respectively. The 25th, 50th, and 75th percentile and maximum concentrations in samples from farms using no antibiotics were 1.0, 2.6, 228.4, and 3,979.2 PFU g $feces^{-1}$, respectively. No significant differences were observed between the levels of strain-RYC2056 phages in samples from both types of farms ($p > 0.05$, generalized Wilcoxon test). Additionally, strain-SP25 phages were present in significantly lower numbers than strain RYC2056 phages when compared within each type of farm ($p = 0.007$ and 0.006, respectively, paired Prentice-Wilcoxon test). In pooled swine fecal samples from farms with antibiotic administration versus those from farms with no use of antibiotics, total coliforms and *E. coli* ranges had similar orders of magnitude: from $10^{5.92}$ to $10^{8.51}$ and from $10^{5.83}$ to $10^{8.40}$ CFU g $feces^{-1}$ versus from $10^{5.55}$ to $10^{8.94}$ and from $10^{5.41}$ to $10^{8.61}$ CFU g $feces^{-1}$, respectively.

Although the concentrations of both bacteriophages were not significantly different in pooled swine feces from

Table 5 | Antimicrobial susceptibility characteristics of bacterial hosts SP25 and RYC2056

	[a]MIC (μg ml^{-1})	
Antimicrobial agent	**SP25**	**RYC2056**
Chlortetracycline	32	0.25
Colistin	>1,024	>1,024
Lincomycin	>1,024	16
Sulfamethazine	>1,024	>1,024
Tiamulin	128	8
Tylosin	1,024	2

[a]Minimal inhibitory concentration.

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farms using antibiotics as compared to those not using antibiotics, the bacterial host strains in the animals' guts may have been affected by antibiotic use. To test this hypothesis, bacterial host strains SP25 and RYC2056 were tested for susceptibility to antibiotics commonly used on swine farms. For chlortetracycline, lincomycin, tiamulin, and tylosin, strain SP25 demonstrated a higher MIC than RYC2056, while both strains exhibited relatively high resistance of more than 1024 $\mu g\ ml^{-1}$ of MIC to colistin and sulfamethazine (Table 5).

Geographical distribution of SP25 phages in pooled swine fecal samples

The occurrence of SP25 phages in partial swine populations was further evaluated by geographic area. SP25 phages were unevenly distributed among farms, with no effect on the part of antibiotic practices (Figure 2). SP25 phages were detected in Pathum Thani, Nakhon Pathom, Chachoengsao, and Suphanburi provinces. In Pathum Thani, Nakhon Pathom, and Chachoengsao provinces, the swine farms containing SP25 phages in their fecal samples were located in close proximity to one another, while the other farms, which did not contain SP25 phages, were in areas adjacent to one another.

DISCUSSION

In this study, *B. fragilis* strains PG76 and RYC2056 isolated from Spain were assessed in comparison to locally isolated strain SP25 in terms of their ability to detect phages specific to swine fecal sources. Strain PG76 was not suitable for use in Thailand due to the absence of phages in

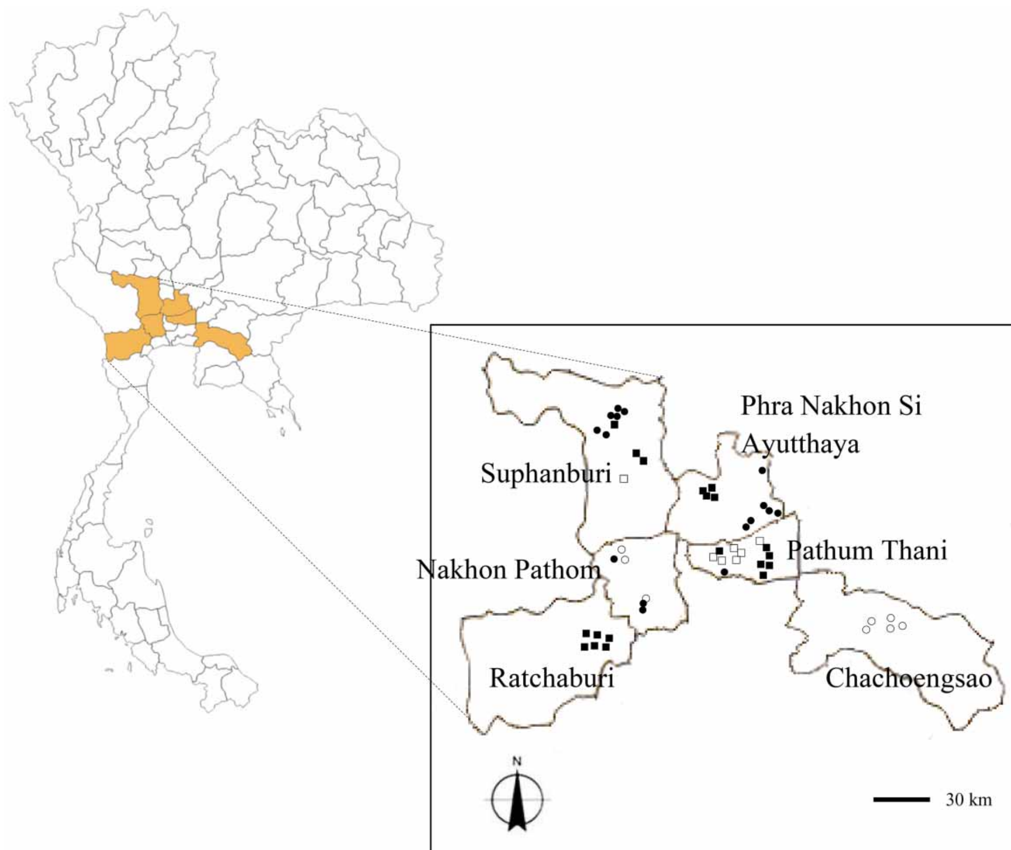

Figure 2 | Geographical distribution of SP25 phages in pooled swine fecal samples; ○, antibiotic swine farms with detectable SP25 phages (higher than 2.6 PFU g^{-1}); ●, antibiotic swine farms with non-detectable SP25 phages (lower than 2.6 PFU g^{-1}); □, non-antibiotic swine farms with detectable SP25 phages (higher than 3.2 PFU g^{-1}); ■, non-antibiotic swine farms with non-detectable SP25 phages (lower than 3.2 PFU g^{-1}).

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pooled swine fecal samples from non-antibiotic farms. RYC2056 phages were detectable in swine fecal samples, but were also found in human sewage, as well as in human-polluted canals. Thus, the independent use of RYC2056 phages as a swine-specific fecal indicator was limited. Variations among geographical regions regarding the specificity of these microorganisms to animal fecal origins and the prevalence of bacteriophages, as observed in this study, have also been previously highlighted (Payan *et al.* 2005; Vijayavel *et al.* 2010; Sirikanchana *et al.* 2014). On the other hand, the local host strain SP25 showed a high specificity to swine fecal contamination, with no cross-detection in the feces of agricultural animals, i.e., sheep, goats, and cattle, or domesticated animals, including cats and dogs. Most importantly, this host strain did not identify phages from any of the human-derived samples being tested. This represented the first reported bacterial strain with 100% specificity to swine fecal origin. Therefore *B. fragilis* strain SP25 demonstrated potential application in differentiation between human fecal pollution and swine fecal pollution.

However, given the partial presence of SP25 phages in swine fecal samples, the presence of strain-SP25 phages could indicate swine fecal pollution, though its absence does not indicate a lack of such contamination. Because we were aware that the bacterial community of the *Bacteroidales* order displays a high level of diversity among pig fecal samples (Jeong *et al.* 2011), we combined more than 30 individual feces into one pooled fecal sample. This was also intended to mimic a real-world contamination situation, in which manure from more than one animal flowing into bodies of water can pose a public health risk. However, variability in the prevalence of *Bacteroides* phages in swine feces was still observed. Bacteriophages of a Gram-negative, coccoid-shaped enterococcus host were also found to have uneven distribution in swine herds (Purnell *et al.* 2011). Moreover, the abundance of SP25 phages in pig feces appeared unrelated to that of the current fecal indicator bacteria (total coliforms and *E. coli*). This study additionally demonstrated that SP25 phages were prevalent in certain groups of farms that were located in close proximity to one another, with no apparent effect on the part of antibiotics administration in the facilities. This suggests that the SP25 host strain could be locally useful for tracking swine fecal pollution in specified districts.

The present study found that *B. fragilis* strain SP25, although isolated from pooled pig feces with no in-feed antibiotic administration, demonstrated a high level of resistance to the antibiotics tested. This was in agreement with a report that showed a high background level of antibiotic-resistant genes in non-antibiotic-fed swine, even though the genes conferred were resistant to antibiotics not administered to the pigs (Looft *et al.* 2012). Information about *Bacteroides*' resistance to antimicrobials commonly used for veterinary purposes is currently limited. It appears that the partial occurrence of phages of strain SP25 in pooled swine fecal samples could be due to a combination of factors, rather than a result of the antimicrobial inactivation effect of bacterial host strains in the gut. Communities of viruses and bacteriophages in swine feces were also reported to be affected by pig age and food but not by enterococcus feeding, such as probiotics (Lu *et al.* 2013; Sachsenröder *et al.* 2014). It was shown that adult pigs contained a higher percentage of *Bacteroides* in their guts than piglets when analyzed via a 454 pyrosequencing technique (Lu *et al.* 2013). Furthermore, pigs fed with synthetic food containing heavy metals as additives exhibited decreased microbial diversity in manures (Lu *et al.* 2013). Moreover, seasonal changes can affect microbial communities, especially in the summer (Merrill & Halverson 2002). Pathogenic infection in pig intestines also affects the intestinal microbial ecosystem (Leser *et al.* 2000). In this study, manures from pigs from each pigsty were mixed to represent pooled samples. Also, pig manures were sampled from December 2012 to June 2014 to cover both the wet and dry seasons. In farms that do not administer antibiotics in feed, pigs were fed with different food types, ranging from spoiled food scraps to instant swine feed. On the other hand, farms that provide antibiotics in feed to pigs tend to use instant swine food. Our preliminary analysis showed that the presence and abundance of phages of strain SP25 did not follow an explicit trend with regard to pig age and season (data not shown).

CONCLUSIONS

The present study provided an insight into variability in the abundance and fecal-source specificity of bacteriophages as

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detected by *B. fragilis* hosts. This study also emphasized the need to characterize the occurrence of phages in local swine populations prior to determining their potential applicability for source tracking in local surface waters.

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