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Verification of *Bacteroidales* 16S rRNA markers as a complementary tool for detecting swine fecal pollution in the Yangtze Delta

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ABSTRACT

To correctly assess and properly manage the public health risks associated with exposure to contaminated water, it is necessary to identify the source of fecal pollution in a watershed. In this study, we evaluated the efficacy of our two previously developed real time-quantitative PCR (qPCR) assays for the detection of swine-associated *Bacteroidales* genetic markers (gene 1–38, gene 3–53) in the Yangtze Delta watershed of southeastern China. The results indicated that the gene 1–38 and 3-53 markers exhibited high accuracy (92.5%, 91.7% conditional probability, respectively) in detecting *Bacteroidales* spp. in water samples. According to binary logistic regression (BLR), these two swine-associated markers were well correlated ($P < 0.05$) with fecal indicators (*Escherichia coli* and *Enterococci* spp.) and zoonotic pathogens (*E. coli* O157: H7, *Salmonella* spp. and *Campylobacter* spp.) in water samples. In contrast, concentrations of conventional fecal indicator bacteria (FIB) were not correlated with zoonotic pathogens, suggesting that they are noneffective at detecting fecal pollution events. Collectively, the results obtained in this study demonstrated that a swine-targeted qPCR assay based on two *Bacteroidales* genes markers (gene 1–38, gene 3–53) could be a useful tool in determining the swine-associated impacts of fecal contamination in a watershed.

Introduction

A variety of enteric bacteria, viruses and protozoan parasites carried by human and animal feces pose a public health risk when they contaminate the water used for drinking, recreation, or crop irrigation (Fu et al., 2014; Nguyen et al., 2018). Traditional regulatory strategies measure fecal indicator bacteria (FIB), including fecal coliforms, *Escherichia coli*, and *Enterococcus* spp., for the prediction of recreational and source water quality (Tambalo et al., 2016; Walters et al., 2007). Even though this method is relatively easy and inexpensive to carry

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out, it requires a long turnaround time (> 24 hr) and is unable to provide information regarding the source of contamination. Notably, FIB usually co-occur with pathogens (Steele et al., 2018), while the relationships between the occurrence of FIB and pathogens in environmental waters remain unpredictable (McQuaig et al., 2012; Corsi et al., 2015; Bradshaw et al., 2016). Understanding the source of contamination is necessary for remediation of polluted waters and thereby taking corrective actions to prevent the transmission of these contaminants.

Microbial source tracking (MST) methods have been applied to provide information about the dominant sources of fecal contamination in environmental waters by detecting host-specific markers (Ahmed et al., 2013; Nguyen et al., 2018). Unlike FIB, *Bacteroidales* markers can identify specific sources of fecal contamination. Comparative studies also have successfully developed qPCR assays based on host-associated *Bacteroidales* DNA markers to identify the source of fecal pollution in water, showing high host specificity and sensitivity (Heaney et al., 2015; Leight et al., 2018; Marti et al., 2011; Odagiri et al., 2015). Moreover, strong correlations between pathogens and host-specific *Bacteroidales* DNA markers have also been reported by several studies (Mulugeta et al., 2012; Frey et al., 2013; Oladeinde et al., 2014), indicating that they can be used as indicators for fecal source tracking. Our previous work (Fan et al., 2017) reported two novel *Bacteroidales* markers-based qPCR assays (genes 1–38 and 3–53) for swine feces-associated bacterial markers in environmental waters. The two markers targeting functional genes exhibited high host-specificity (> 90%) in quantitative PCR assays with 71 fecal DNAs from non-target animal sources. Considering the variation in the host specificity results, validation of MST markers against a panel of environmental water samples has been recommended (Field and Samadpour, 2007; Harwood et al., 2014). Moreover, little is known about their survival and correlation with pathogens.

Thus, the present study evaluated the ability of two swine-associated bacterial (gene 1–38, gene 3–53) markers to discriminate and measure swine feces contamination in environmental water samples of the Yangtze Delta watershed. The water samples were also tested for FIB and three important potential waterborne pathogens. Moreover, the study aimed to assess the relationships between MST markers and pathogen types, yielding potentially valuable information for subsequent mitigation efforts.

1. Materials and methods

1.1. Sample collection and DNA extraction

A total of 64 water samples were collected between October 2015 and September 2018 from 27 different geographic regions in the Yangtze Delta of Southeastern China (Fig. 1). Agricultural farming activities are common in the watershed area. The suspected sources of fecal pollution within the study area include application of stored manure, animals on farms, septic systems, effluent release from municipal lagoons, and wildlife. An approximately 15-L water sample was collected from each site in sterile containers at 30 cm

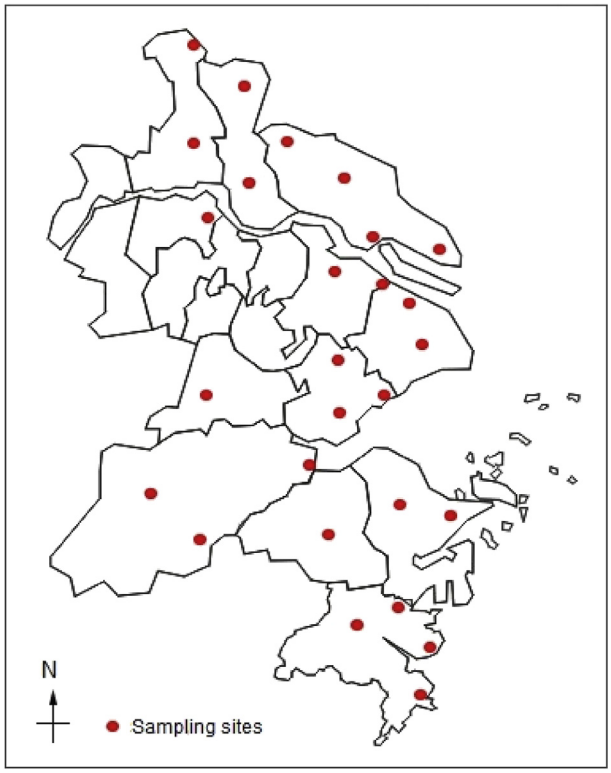

Fig. 1 – Map of the Yangtze Delta showing sampling sites.

below the water surface. Samples were transported to the laboratory at 4°C and processed within 6–8 hr. Each sample was filtered by 0.45 μm polycarbonate membranes (CN-6 Metricel® Grid 47 mm, life Science). Membranes were then immediately placed in sterile conical tubes for DNA extraction and stored at −80°C until use. For qPCR analysis of swine-associated *Bacteroidales* genetic markers (gene 1–38, gene 3–53) and bacterial pathogens, all DNA extractions were obtained with the QIAamp® Fast DNA Stool Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA concentrations were determined using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Thermo Fisher).

1.2. Enumeration of fecal indicator bacteria (FIB)

The membrane filtration method was used to process water samples for the enumeration of FIB. 100 mL portions of the water samples were 10-fold serially diluted with sterile MilliQ water and filtered through membranes (0.45 μm pore size). These were then placed on modified membrane-thermotolerant *Escherichia coli* agar (mTEC agar) (Hopebio, Qingdao, China) and membrane-*Enterococcus* indoxyl-*D*-glucoside (mEI) agar (Hopebio, China) for the isolation of *E. coli* and *Enterococci* spp., respectively. Modified mTEC agar plates were incubated for 2 hr at 35°C to recover stressed cells. This was followed by incubation for 22 hr at 44°C (U. S. Environmental Protection Agency, 1997.), and the mEI agar plates were incubated at 41°C for 24 hr (U. S. Environmental Protection Agency, 2002). The viable *E. coli* and *Enterococci* spp. counts were expressed as the average logarithmic value of three replications for each sample.

1.3. *Detection of swine-associated* Bacteroidales *in water samples by qPCR assays*

A primer set targeting functional genes of *Bacteroidales* (gene 1–38, gene 3–53) for qPCR assay was used to detect swine fecal contamination in samples (Table 1). Positive controls for gene 1-38- and 3-53-based qPCR assays were the plasmid DNA containing 1–38 and 3–53 DNA fragments, respectively. The 1–38 or 3–53 DNA fragment was derived from potential swine-associated metagenomic DNA fragments enriched by the Genome fragment enrichment (GFE) method. The limits of quantitation (LOQ) of gene 1-38- and 3-53-based qPCR assays were determined to be 240 and 24 DNA copies (Fan et al., 2017). Prior to the qPCR, a *Bacteroides* HF183 marker-based general assay was conducted to determine the potential for PCR inhibition in DNA extracts from all water samples (Ahmed et al., 2013). All DNA samples were spiked with 10^3 copies of the HF183 marker. The Ct values of the spiked DNA extracts detected by qPCR assays were compared with those of the distilled water spiked with the same number of HF183 markers to confirm the absence of PCR inhibition.

The gene 1-38- and 3-53-based qPCR assays were performed in 20 μL reaction mixtures using Premix Ex Taq™ (Probe qPCR) Supermix (Takara, Dalian, China). The PCR mixture contained 10 μL of Supermix, 400 nmol/L of each primer (Table 1), 400 nmol/L of each corresponding probe (Table 1), and 5 μL of template DNA. No-template reactions were used as negative controls for each PCR experiment. The real-time qPCR assays were performed on a Lightcycler® 480 II (Roche, UK). MST markers were $\log_{10}$ transformed.

1.4. *Detection of zoonotic pathogens in water samples by qPCR assays*

Three common pathogens, including *E. coli* O157:H7, *Salmonella enterica* serovar *Typhimurium*, and *Campylobacter jejuni*, were used for detection of pathogens in this study. They were quantitated by the analysis of *E. coli O157 rfbE*, *Salmonella invA* genes, and *Campylobacter* 16S rRNA with qPCR assays. Positive controls for these qPCR assays were the genomic DNA isolated from *E. coli* O157:H7 ATCC 35150, *Salmonella enterica* serovar *Typhimurium* ATCC 14028, and *Campylobacter jejuni* NCTC 11168, respectively. The standard curves of the qPCR assays were drawn up with ten-fold serial dilutions of DNA standards in ranging from 2×10^6 to 2×10^0 copies/μL. The LOQ of each assay was considered as the lowest amount of DNA standard that could be detected in all repeats.

The qPCR assays of *Campylobacter* 16S rRNA and *E. coli* O157 *rfbE* were performed in 20 μL reaction mixtures using Premix Ex Taq™ (Probe qPCR) Supermix (Takara, Dalian, China). The PCR mixture contained 10 μL of Supermix, 400 nmol/L of each primer (Table 1), 400 nmol/L of each corresponding probe (Table 1), and 5 μL of template DNA. The *Salmonella invA* amplifications were performed in 20 μL reaction mixtures using SYBR®Green Premix Ex Taq™ Supermix (Takara, Dalian, China). The PCR mixture for the assay comprised 10 μL of Supermix, a 400 nmol/L concentration of each primer (Table 1), and 5 μL of template DNA. The qPCR assays were performed on a Lightcycler® 480 II (Roche, UK). The pathogen data were $\log_{10}$ transformed. For each assay, three independent biological replicates were used (Intra-assay repeatability). Each replicate was repeated 10 times (technical replications) resulting in inter-assay reproducibility. No-template reactions were used as negative controls.

1.5. *Conditional probability analysis using Bayes theorem*

The detection of swine feces-associated markers in water samples that were contaminated with swine feces rather than the non-target fecal materials was expressed as the conditional probability. The method used was called Bayes' theorem, which has been previously described (Kildare et al., 2007; Weidhaas et al., 2011). Calculation of $P(H \setminus T)$, the probability (P) that there is swine feces contamination (H) in a water sample given the event that the swine-associated markers are positive (T) for the water sample, is shown in the following equation: $P(H \setminus T) = [P(T \setminus H)\ P(H)] \setminus [P(T \setminus H)\ P(H) + P(T \setminus H')\ P(H')]$, where $P(T \setminus H)$ is the probability of detecting the swine-associated

Table 1 – Sequences of primers and probes used for real-time PCR assays.

Marker or organism in qPCR assay	Target	Primer or probe sequence (5′-3′)[a]	Amplicon size (bp)	Reference
1–38	Information store and processes	GGAGGTGGTTAAGCCGATATGTT GCCCCTTTCTTGATACTTTGGA Fam-AAACTGATTGGAGAAGAATACAGGCG-Tam	119	Fan et al. (2017)
3–53	Information store and processes	GCGTCGTTACATCCTCGAAAG GCGTTTGGGCTTGAATGG Fam-TTCACGCATTATGGTGTGCGATGATGCAA-Tam	124	Fan et al. (2017)
E. coli O157	*rfbE*	GCAGATAAACTCATCGAAACAAGG CGATAGGCTGGGGAAACTAGG TET- TCCACGCCAACCAAGATCCTCAGC-TAMRA	141	
Campylobacter spp.	16S rRNA	CAC GTG CTA CAA TGG CAT AT GGC TTC ATG CTC TCG AGTT FAM-CAG AGAA CAA TCC GAA CTG GGA CA-BHQ1	108	Lund et al. (2004)
Salmonella spp.	*invA*	ACA GTG CTC GTT TAC GAC CTG AAT AGA CGA CTG GTA CTG ATC GAT AAT	244	Chiu and Ou (1996)

[a] F, forward primer; R, reverse primer; P, probe; Fam, 6-carboxyfluorescein; Tam, 6-carboxytetramethylrhodamine; BHQ, black hole quencher.

markers in a water sample impacted by swine feces (true positive); $P(T \setminus H')$ represents the probability of detecting the swine-associated markers in a water sample not impacted by swine feces (false positive); and $P(H)$ is the background probability that a marker is detected in a water sample, respectively, while $P(H')$ is the background probability of detecting a marker that is absent from a water sample. The value of $P(H')$ is 1- $P(H)$.

1.6. Correlation analysis

Pearson's correlation (r_p) was calculated to determine the relationship between *E. coli* and *Enterococcal* numbers in water samples. A binary logistic regression (BLR) (SPSS version 20.0) analysis was also performed to obtain correlations between swine feces-associated markers and the distribution of FIB and zoonotic pathogens. This is a commonly used technique to model the binary (presence/absence) result from water samples. The presence/absence of swine feces-associated markers and zoonotic bacterial pathogens was treated as the dependent variable (a binary variable). The value of a target organism present/absent was assigned 1/0, respectively. Correlations among FIB, swine feces-associated markers and zoonotic bacterial pathogens were considered significant when the P value for the model chi-square was < 0.05. The odds ratio (OR) represents the degree of change in the dependent variable with a change in the independent variable, and the confidence interval for the odds ratio did not include 1.0.

1.7. Statistical analysis

Linear regression analysis, t-tests, and analysis of variance (ANOVA) tests were performed using Origin 8.0 and IBM SPSS statistics 20 software.

2. Results and discussion

2.1. PCR inhibitors

For the HF183-spiked distilled water, the Ct value for human-specific *Bacteroides* DNA was 27.5 ± 0.04. For the HF183-spiked undiluted DNA isolated from water samples of JC, CX, ZDT, ZJ, XS, YC area, the Ct values ranged from 30.4 to 35.6 (Table 2). Based on the result of ANOVA analysis, the test samples had significantly higher Ct values than the HF183-spiked distilled water, which demonstrated that undiluted DNA samples could not be used for the PCR assays directly due to the presence of potential PCR inhibitors. PCR inhibitors in water DNA samples could mask PCR amplification and further induce false positive/negative results. Therefore, to quantify the host-specific markers and pathogen accurately, it is very necessary to remove these inhibitors by DNA dilution (Ahmed et al., 2010). The Ct values of these HF183-spiked diluted DNAs ranged from 27.3 to 28.9 (Table 2) once they were 10-fold diluted. Their obtained Ct values had no significant difference ($P > 0.05$) from the Ct values of the spiked distilled water. This indicated that no PCR inhibitor was observed in these10-fold diluted test DNAs.

For water samples collected from LA, JH, HZ, HS, HZE, LX, JD, ZRF, XX, HK, JZXW, MZ, PY, XNH, SX, JP, LLC, TT, CX, DR areas, all the mean Ct values ranged from 27.6 to 28.4, and no significant difference ($P > 0.05$) was observed between the mean Ct values of the spiked distilled water and undiluted DNAs, indicating that the test samples collected from these areas were free of PCR inhibitors. These water DNAs were able to be used for detection directly.

Table 2 – Evaluation of PCR inhibition on the PCR detection of spiked sewage-associated HF183 *Bacteroides* markers in DNA isolated from water samples as opposed to distilled water samples.

DNA sample source	No. of samples	Ct value (range) of real-time PCR	
		Undiluted DNA	10-fold dilution[a]
Distilled water	1	27.5 ± 0.04	ND[b]
JC	6	30.8–34.1	27.9–28.3
CX	2	30.4–35.5	27.7–28.2
ZDT	2	30.6–35.4	27.9–28.3
ZJ	1	32.7 ± 0.03	27.3 ± 0.06
SX	4	31.7–32.5	28.0–28.4
YC	2	30.8–35.6	27.7–28.9

[a] These test samples had higher Ct values than the HF183-spiked distilled water, which demonstrated undiluted DNA samples could not be used for the PCR assays directly due to the presence of potential PCR inhibitor, serial 10-fold dilution was performed;
[b] ND, 10-fold dilution was not performed.

2.2. qPCR standard curves and limit of quantification

Three different standard quantification curves for *C. jejuni*, *E. coli* O157:H7, and *S. Typhimurium* were created as aforementioned. All three assays exhibited a strong linear relationship ($r^2 > 0.99$), and all the amplification efficiencies of the three assays were > 95% (Fig. 2). Some previous studies have also reported the excellent amplification efficiencies of these three standard curves, which ranged from 96% to 100% (Ahmed et al., 2013; Shanks et al., 2011). The amplification efficiencies of gene 1–38 and gene 3–53 based assays were > 94%, and the correlation coefficient was > 0.99 (Fan et al., 2017). The qPCR limit of

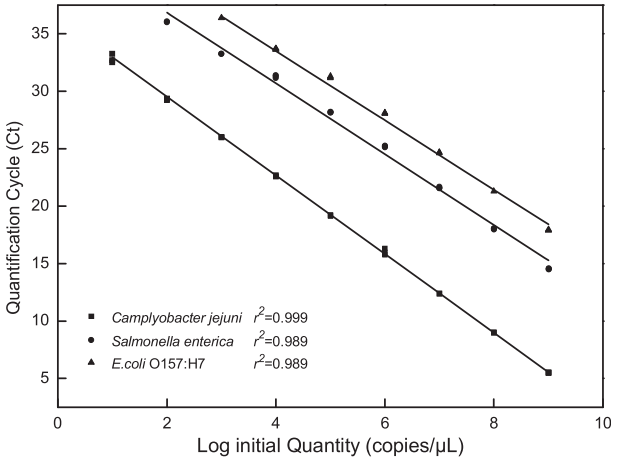

Fig. 2 – Standard curves of the TaqMan real-time PCR assays for quantification of zoonotic pathogens.

quantification was as low as 4 gene copies, 2 gene copies and 3 gene copies for *C. jejuni*, *E. coli* O157:H7, and *S. Typhimurium* assays, respectively. These results implied that these assays could be reliably applied to quantify the pathogens within the quantification range of seven orders of magnitude.

2.3. *Quantification of FIB, swine feces-associated markers and pathogens of water samples*

Among the 64 samples tested, 81.3% and 87.5% of water samples were found to contain *E. coli* and *Enterococcus* spp., with the concentrations of 0.48–4.77 and 0.22 to 5.01 Log CFU/100 mL, respectively (Table 3). Pearson's correlation analysis found no significant correlation between *E. coli* and *Enterococcus* spp. ($r_p = 0.278$; $P = 0.061 > 0.05$). Also, the average concentration of *Enterococcus* spp. exceeded that of *E. coli* in most test samples.

Of the 64 water samples tested, 90.6% of water samples (58 samples) were collected from watersheds possibly impacted by a local swine farm. However, 51.6% and 53% samples were PCR-positive for gene 1–38 and gene 3-53 markers, respectively (Table 3). This indicated that not all the watersheds near swine farms were contaminated by feces. Moreover, these two markers were not detected simultaneously in some water samples. The inconsistency between the two markers in water was attributed to the differences in their specificity and sensitivity (Nguyen et al., 2018). There were 28 samples and 31 samples that were true positive for gene 1–38 and gene 3–53, respectively (Table 3). In addition, these two swine-associated markers occurred in some water samples collected from the watersheds near non-target animal farms (Table 3). These false positive results indicated that it is difficult to design a PCR assay that could be absolutely host-specific and discriminate all possible hosts of fecal *Bacteroidales* (Ahmed et al., 2013). The abundance of *Bacteroidales* markers (gene 1–38 and gene 3–53) varied significantly among sampling sites, with gene copy numbers of 2–4, 1 to 2 Log copies/μL, respectively (Data not shown). However, no significant variation for *Bacteroidales* concentrations was found by sampling date, which is consistent with the previous study by Drozd et al. (2013). Concentration of larger amounts of water will increase the concentration of markers

Table 3 – Concentration of *E. coli* and *Enterococcus* spp. and real-time PCR positive/negative results of swine feces-associated markers and pathogens in water samples collected from southeastern China.

Sampling sits	No. of Samples	Fecal indicator range (avg), Log10 CFU per 100 mL		No. of samples with positive PCR results for:				
		E. coli	*Enterococcus* spp.	1–38	3–53	*E. coli* O157 *RfbE* gene	*Campylobacter* 16S rRNA	*Salmonella invA* gene
JC	6	0.00–2.38 (1.50)	0.00–2.82 (2.53)	3	3	3	0	3
LA[b]	6(1)	0.00–4.77 (4.05)	0.00–5.01 (4.48)	2	2	3	1	2
JH	4	0.00–1.13 (0.67)	0.00–2.15 (2.14)	0	1	1	0	1
HZ[b]	6(1)	0.00–1.31 (0.53)	0.00–2.70 (2.68)	1	2	2	1	2
HS	4	0.00–1.31 (1.19)	0.00–2.12 (2.09)	1	1	1	0	1
CX[a]	2(1)	0.00–3.78 (2.64)	0.00–2.17 (1.18)	1	2	2	1	2
HZE[a]	2(2)	0.00–4.25 (2.99)	0.00–3.50 (2.70)	1	1	2	0	1
LX[a]	1(1)	0.00–2.57 (2.53)	0.00–2.00 (1.95)	1	0	1	0	0
JD	1	0.00–1.45 (0.54)	0.00–1.67 (0.79)	1	1	0	1	1
ZDT	2	0.00–3.38 (2.95)	0.00–2.21 (1.09)	2	1	2	0	1
ZRF	2	0.00–1.05 (0.48)	0.00–2.11 (1.05)	1	2	2	2	1
XX	1	0.00–2.65 (2.61)	0.00–1.26 (0.45)	1	1	1	1	0
HK	1	0.00–1.40 (1.36)	0.00–2.53 (2.42)	1	0	1	0	1
JZ	1	0.00–2.36 (2.29)	0.00–1.17 (0.57)	1	0	1	1	1
XW	1	0.00–2.65 (2.58)	0.00–2.85 (2.70)	1	1	1	0	1
MZ	3	0.00–4.19 (3.01)	0.00–2.26 (1.33)	2	3	3	2	3
PY	4	0.00–4.31 (2.41)	0.00–2.59 (1.89)	4	4	4	0	2
ZJ	1	0.00–1.35 (0.62)	0.00–2.26 (2.23)	1	1	1	0	1
XNH	3	0.00–3.00 (2.95)	0.00–1.78 (1.07)	3	1	3	0	3
SX	4	0.00–4.20 (3.17)	0.00–2.16 (1.95)	1	1	2	1	2
JP	1	0.00–1.32 (0.46)	0.00–1.54 (1.44)	0	1	1	0	1
XS	1	0.00–1.54 (0.76)	0.00–0.78 (0.67)	1	1	1	0	0
LLC	1	0.00–1.78 (0.78)	0.00–3.08 (3.03)	1	1	1	0	1
TT	2	0.00–3.45 (3.45)	0.00–3.36 (2.71)	0	2	2	0	0
CX	1	0.00–1.58 (0.82)	0.00–1.70 (1.53)	0	1	1	0	0
YC	2	0.00–2.56 (2.53)	0.00–1.36 (1.03)	1	0	2	0	1
DR	1	0.00–1.57 (1.51)	0.00–3.37 (3.35)	1	0	1	0	1
Total	64(58)[c]			33(28)[d]	34(31)[d]	45	11	33

[a] A part of sampling sites nearby duck farm and goat farm, respectively. Numbers in parentheses represent the number of sampling sites nearby duck farm and goat farm, respectively; [b] A part of sampling sites nearby duck farm and goat farm, respectively. Numbers in parentheses represent the number of sampling sites nearby duck farm and goat farm, respectively; [c] 58 water samples collected from sites near swine farms; [d] The number of true positives for water sample DNA.

and LOD, further improving source tracking (Griffin et al., 2001).

Waterborne pathogen occurrences in water varied by sampling site (Table 3). *E. coli* O157 and *Salmonella* were ubiquitous in the 64 samples tested, with detection rates of 70.3% and 51.5%, with the concentrations of 0.43–5.12 and 0.52 to 4.37 Log CFU/100 mL, respectively. However, *Campylobacter* was detected in 17.2% of samples, with the concentration of 0.71–2.73 Log CFU/100 mL. *Salmonella* and *Escherichia coli* O157 persist longer in the environment than *Campylobacter* (Frey et al., 2013), making the detection of *Campylobacter* less frequent. Ahmed et al. (2013) also reported that the occurrence rates of *Salmonella* and *Escherichia coli* O157 were higher than that of *Campylobacter* in composite bovine wastewater.

2.4. The conditional probability of accurately detecting swine fecal contamination

Bayes' theorem has been used to analyze the probabilities of correctly detecting host feces associated markers in environmental samples (Kildare et al., 2007; Weidhaas et al., 2011; Ryu et al., 2012). In this study, Bayes' theorem was employed to detect swine feces pollution, which is essential to minimize public health risks from pathogen exposure in the watershed of the Yangtze Delta, China. The background probabilities, $P(H)$, of detecting the gene 1–38 and 3-53 markers in the water samples by qPCR were 0.48 and 0.53, respectively. Moreover, the background probabilities that these markers were not detected in the water samples were 1-$P(H)$, 0.52 (gene 1-38 marker) and 0.47 (gene 3-53 marker). $P(T \setminus H)$ is the probability of detecting the swine specific markers in an environmental sample impacted by swine feces (true-positive rate), and the values were determined from the host sensitivity assays as previously reported (0.94 for 1-38 marker, 0.99 for 3-53 marker) (Fan et al., 2017). The swine specificities of the 1-38 marker-based assays and 3-53 marker-based assay were 93% and 10%, respectively (Fan et al., 2017). Therefore, $P(T \setminus H')$, the probability of detecting the swine-specific markers in an environmental sample impacted by non-target source of feces (false-positive rate), was calculated as 0.07 for the 1-38 marker and 0.10 for the 3-53 marker. The conditional probabilities of accurately detecting swine fecal contamination in water by the 1-38 marker-based assays and 3-53 marker-based assays were 92.5% and 91.7%, respectively. These results demonstrated the practical effectiveness of the 1-38 marker-based assays and 3-53 marker-based assays. This also validated the two previously developed assays in natural water samples and proved the difficulty in discriminating swine and other animals' fecal waste by molecular analysis. Moreover, some studies reported that a larger number of water samples needs to be investigated and a combination of MST markers would be required to obtain confirmatory results (Ahmed et al., 2012; Oladeinde et al., 2014).

2.5. Correlations among FIB, swine feces-specific markers and pathogens of water samples

A pairwise comparison analysis was performed to determine the relationship between the occurrences of swine feces-specific markers with FIB and pathogens in water samples (Table 4). The concordance was obtained by adding the percentage of co-occurrence and non-co-occurrence for each pairwise comparison. For the comparisons between swine-associated markers and FIB, the 1-38 markers had higher concordance with *E. coli* (60.9%) and *Enterococcus* spp. (62.5%) than the 3-53 markers (Table 4). In addition, swine-associated markers had a significantly higher concordance with pathogens than FIB. *Enterococcus* spp. and *E. coli* O157 *rfbE* gene had the concordance of 71.9%, while FIB had low concordance (31.3%, 14.1%) with *Campylobacter* 16S rRNA. Consistently, many previous studies demonstrated that FIB had poor concordance with pathogens, while *Bacteroidales* markers could be well accordant with pathogens (Ahmed et al., 2013; Frey et al., 2013; Bradshaw et al., 2016).

Table 4 – Relationship among swine-feces- associated markers, FIB, and pathogens in water samples using binary logistic regression analysis.

Comparison	Concordance (%)	Odds ratio	P value[a]
1-38 vs. *E. coli*	60.9	0.98	0.618
1-38 vs. *Enterococcus* spp.	62.5	0.99	0.175
1-38 vs. *E. coli* O157 *RfbE*	75.0	0.98	0.984
1-38 vs. *Salmonella invA*	31.3	1.72	0.050
1-38 vs. *Campylobacter* 16S rRNA	76.6	1.00	0.817
3-53 vs. *E. coli*	35.9	0.99	0.299
3-53 vs. *Enterococcus* spp.	29.7	1.00	0.322
3-53 vs. *E. coli* O157 *RfbE*	76.6	0.98	0.050
3-53 vs. *Salmonella invA*	54.7	1.00	0.438
3-53 vs. *Campylobacter* 16S rRNA	73.4	0.98	0.177
E. coli vs. *E. coli* O157 *RfbE*	46.9	0.99	0.131
E. coli vs. *Campylobacter* 16S rRNA	31.3	1.00	0.331
E. coli vs. *Salmonella invA*	53.1	0.99	0.390
Enterococcus spp. vs. *E. coli* O157 *RfbE*	71.9	1.00	0.764
Enterococcus spp. vs. *Campylobacter* 16S rRNA	14.1	1.00	0.609
Enterococcus spp. vs. *Salmonella invA*	62.5	0.98	0.442

[a] The *P* value for the model chi-square was ⟨ 0.05, and the confidence interval for the odds ratio did not include 1.

BLR analysis indicated that there was no correlation between the concentration of FIB and the presence/absence of swine-specific markers and zoonotic pathogens (Table 4). This may be attributed to the fact that the molecular signal of swine feces-associated markers and pathogens persists considerably longer compared to culturable organisms (FIB) (Oladeinde et al., 2014). Moreover, this result is consistent with the correlation tests shown in Fig. 3. When the concentration of *E. coli* ranged from 0 to 1 Log CFU/100 mL, the swine-associated markers and pathogens gene were measured with high rates of positive detections, except for *Campylobacter* 16S rRNA. When the concentration of *E. coli* was > 4 Log CFU per 100 mL, the swine-associated markers and pathogen genes were rarely detected, and even *Campylobacter* 16S rRNA could not be detected by qPCR assay. Similarly, when the concentration of *Enterococcus* spp. ranged from 3 to 4 Log CFU/100 mL, no *Campylobacter* 16S rRNA was detected by qPCR assay. Also, 3-53 marker, *E. coli* O157 *rfbE*, *Campylobacter* 16S rRNA were not detected in water sample DNAs when the

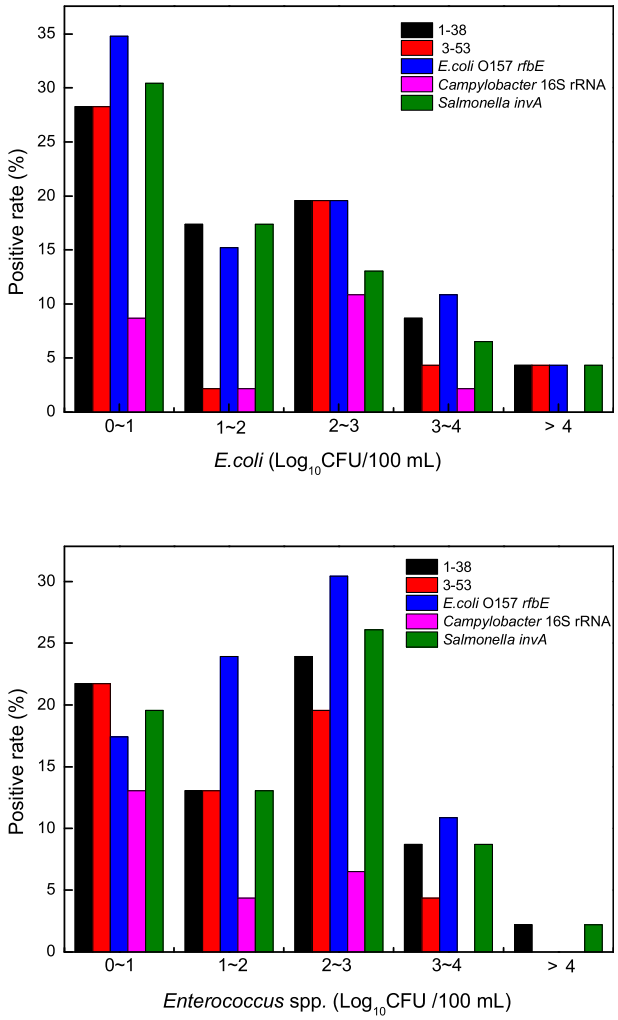

Fig. 3 – Relationship between concentration of FIB and the positive detection rate of swine-associated markers and pathogens.

concentration of *Enterococcus* spp. was > 4 Log CFU/100 mL. Collectively, this study showed that FIB detection was unreliable at predicting the presence of specific waterborne pathogens, which is in agreement with previous research (Pachepsky et al., 2006; Jokinen et al., 2012).

Swine feces-associated markers had a high concordance with *E. coli* O157 *rfbE* and *Salmonella invA* (Table 4), suggesting that swine feces-associated markers may have the potential to indicate the occurrence of potential pathogens. The gene 1-38 marker was positively correlated with the occurrence of *Campylobacter* 16S rRNA in water ($P = 0.04$; OR = 1.72), and the gene 3-53 marker was also positively correlated with *E. coli* O157 *rfbE* ($P = 0.04$; OR = 0.98). However, there is no correlation between the occurrence of swine feces-associated markers in water with other pathogens (Table 4). Wilkes et al. (2011) reported that the prevalence of waterborne pathogens is related to seasonal attributes. Therefore, some essential parameters (i.e. seasonal variability) factors should be considered during microbial water quality assessment in further research.

3. Conclusion

Swine feces-associated *Bacteroidales* markers (1-38 marker, 3-53 marker) were evaluated by the detection and quantitation of fecal contamination in natural waters. The results concluded that these markers are a potentially valuable tool in monitoring programs and identifying sources of fecal pollution in environmental water. The application of multiple swine feces-associated markers is recommended to confirm the evidence of fecal pollution rather than a single marker. The concentrations of FIB were not correlated with swine feces-associated markers or zoonotic pathogens, which suggested that FIB applied alone could not obtain sufficient information on the microbiological quality of water. In contrast, the *Bacteroidales* markers (1–38 makers, 3-53 marker) exhibited correlation with pathogens in water samples, indicating that these markers have the potential to predict pathogens in an aquatic ecosystem. Therefore, a combination of FIB, multiple MST markers and water quality measurements should be employed to assess microbial water quality and predict human health risks from exposure to feces-contaminated water. Further research will be required to understand the persistence of these markers in environmental water.

Conflict of interest

The authors declare that they have no conflict of interest.

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