Front. Environ. Sci. Eng. 2020, 14(4): 67
https://doi.org/10.1007/s11783-020-1246-9

RESEARCH ARTICLE

Validation of *Bacteroidales*-based microbial source tracking markers for pig fecal pollution and their application in two rivers of North China

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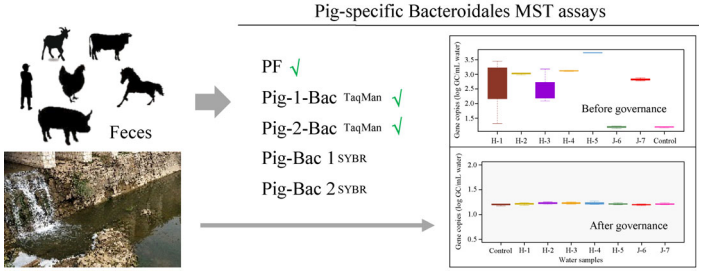
HIGHLIGHTS

- Pig feces is the predominant excrement produced by animal husbandry in China.
- The PF, Pig-1-Bac^{TaqMan}, and Pig-2-Bac^{TaqMan} MST assays showed better performance.
- The pig-specific MST assays can contribute to managing the pig fecal pollution.

GRAPHIC ABSTRACT



ARTICLE INFO

Article history:
Received 16 January 2020
Revised 24 February 2020
Accepted 11 March 2020
Available online 15 April 2020

Keywords:
Microbial source tracking
Pig fecal pollution
16S rRNA gene markers
Pig-specific *Bacteroidales*

ABSTRACT

In China, pig feces is the predominant source of excrement produced by animal husbandry. Improper use or direct discharge of pig feces can result in contamination of natural water systems. Microbial source tracking (MST) technology can identify the sources of fecal pollution in environmental water, and contribute to the management of pig fecal pollution by local environmental protection agencies. However, the accuracy of such assays can be context-dependent, and they have not been comprehensively evaluated under Chinese conditions. We aimed to compare the performance of five previously reported pig-specific MST assays (PF, Pig-Bac1^{SYBR}, Pig-Bac2^{SYBR}, Pig-1-Bac^{TaqMan}, and Pig-2-Bac^{TaqMan}, which are based on *Bacteroidales* 16S rRNA gene markers) and apply them in two rivers of North China. We collected a total of 173 fecal samples from pigs, cows, goats, chickens, humans, and horses across China. The PF assay optimized in this study showed outstanding qualitative performance and achieved 100% specificity and sensitivity. However, the two SYBR green qPCR assays (Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR}) cross-reacted with most non-pig fecal samples. In contrast, both the Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays gave 100% specificity and sensitivity. Of these, the Pig-2-Bac^{TaqMan} assay showed higher reproducibility. Our results regarding the specificity of these pig-specific MST assays differ from those reported in Thailand, Japan, and America. Using the PF and Pig-2-Bac^{TaqMan} assays, a field test comparing the levels of pig fecal pollution in rivers near a pig farm before and after comprehensive environmental pollution governance indicated that pig fecal pollution was effectively controlled at this location.

1 Introduction

In China, there were about 430 million pigs in 2016 (Huang, 2017), and pig feces accounts for the largest proportion of the excrement produced by China's animal husbandry. According the statistical yearbook, pig fecal waste in 2015 reached 1.77 billion tons, significantly higher than that produced by poultry (89 million tons) and the tens of millions of tons discharged by cows, horses, and other livestock (Li and Song, 2018). Fecal waste generated by pig husbandry in China is normally stored in septic tanks or used as fertilizer, but occasional leakages and direct discharge pose a high risk to environmental waters. In view of this, China's environmental protection agency is urgently seeking effective means to accurately identify the source of pig fecal pollution in environmental water.

Microbial source tracking (MST) technology provides

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effective means for the identification of fecal sources in environmental waters by detecting specific gene markers or physio-biochemical features of host-specific gut microorganisms such as *Bacteroides* spp. in *Bacteroidales*, *Bifidobacterium* spp. and some viruses (Harwood et al., 2014; Gao and Tao, 2012). Currently, many MST assays use the host-specific 16s rRNA gene marker of *Bacteroidales* as an indicator. This has successfully discriminated the source of human and animal fecal pollution in a wide range of geographic regions including Nepal (Malla et al., 2018), Ireland (Dorai-Raj et al., 2009), Canada (Wilkes et al., 2013), Tanzania (Mattioli et al., 2013), and New Zealand (Green et al., 2012). However, since the gut community in humans and animals is susceptible to the changing of food and other environmental conditions, the applicability of MST assays exhibits regional differences (Nshimyimana et al., 2017). For example, the specific gene marker HF183 of *Bacteroidales* is recommended as an indicator of human fecal pollution in the United States (Layton et al., 2013), but is not suitable for determining human pollution in India (Odagiri et al., 2015). Similarly, Odagiri et al. (2015) found that the *Bacteroidales* gene marker BacHum, which could effectively differentiate human fecal pollution in India, was not sensitive enough in Singapore (Nshimyimana et al., 2017). Therefore, it's necessary to validate the performance of *Bacteroidales*-associated MST assays in specific regions before using them to discriminate the sources of fecal pollution in environmental waters.

In this study, we evaluated the performance of five pig-specific MST assays that use *Bacteroidales* 16S rRNA gene markers (PF, Pig-Bac1, Pig-Bac2, Pig-1-Bac, and Pig-2-Bac) as indicators. The PF assay was selected based on performance comparison of 41 MST assays in 27 laboratories (Boehm et al., 2013) for qualitative analysis (Bernhard and Field, 2000; Dick et al., 2005). The Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays, which use quantitative PCR with the DNA binding dye SYBR green, have been validated in Japan (Okabe et al., 2007), but are rarely applied in other regions. Two other quantitative assays, Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan}, which are based on dual-labeled hydrolysis TaqMan probes, were also selected for their high specificity (Mieszkin et al., 2009). Notably, regional differences among these assays have been reported in previous studies (Gourmelon et al., 2007; Fremaux et al., 2009). For example, Malla et al. (2018) found that the PF assay had a specificity of only 57% in Nepal, which was quite different from that reported in France (>90%) (Gourmelon et al., 2007). Currently in China, reports of the validation and application of these pig-specific MST assays are rather scarce. The objectives of this study were 1) to validate the performance of the five selected pig-specific *Bacteroidales* assays by testing animal and human fecal samples and 2) to carry out field testing to evaluate pig fecal pollution in two rivers of North China before and after comprehensive environmental pollution governance.

2 Materials and methods

2.1 Sample collection

2.1.1 Fecal samples

During 2018, we collected fecal samples from Guangxi (20°–26°N, 104°–112°E), Henan (31°–36°N, 113°–116°E), Beijing (39°–41°N, 115°–117°E), and Inner Mongolia (47°–52°N, 119°–122°E). Approximately 15–20 g of fresh feces was sampled from humans and different animals using 50 mL sterile centrifuge tubes (Corning Inc., Tewksbury, MA USA) and frozen on ice. The collected fecal samples were then sent back to the laboratory as soon as possible and stored at –80°C. A total of 173 fresh fecal samples was collected from pigs (n = 80), cows (n = 23), goats (n = 25), chickens (n = 21), humans (n = 20), and horses (n = 4).

2.1.2 Environmental water samples

Environmental water sampling was conducted in two rivers (Fig. 1). There is a pig farm near River H that stores sewage water in septic tanks. Five sites in River H were selected for sampling upstream and downstream of the pig farm. Considering that River H flows into River J, River J may be contaminated by pig feces. We therefore also sampled in River J, upstream and downstream of the confluence. At each site, we sampled twice in October 2018 and April 2019 respectively, during which comprehensive environmental pollution governance was conducted by the local environmental protection agency. This mainly consists of plugging the sewage outlets along the rivers and shutting down the pig farms that fail to meet the

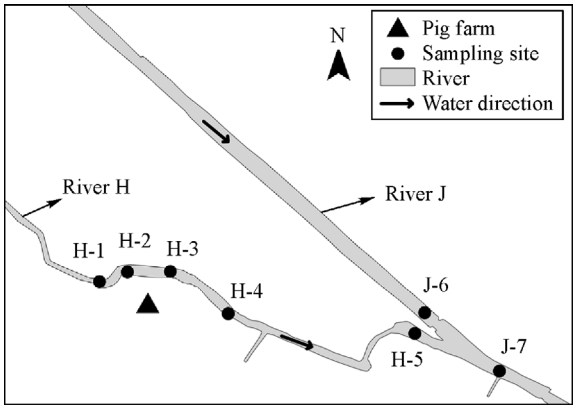

Fig. 1 Sampling locations for environmental waters.

discharge standards. There was no rain for at least one week before each sample collection. At each sampling site, the flow velocity of the river water at two sampling times was similar, and the water temperature was around 15°C.

Water samples of approximately 500 mL were collected in triplicate from each site and placed in a sterile bottle. All samples were stored in incubators with ice and sent back to the laboratory with minimal delay. After filtering with 0.45μm pore-size filters (Merck Millipore, Billerica, MA, USA), the filters were sliced using a flame-sterilized scalpel and stored at –80°C. Meanwhile, three bottles of distilled water (each 500 mL) were filtered as controls.

2.2 Genomic DNA extraction

Genomic DNA of all fecal and environmental water samples was extracted using a FastDNA™ Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's recommendations. Briefly, about 0.4 g of each fecal sample or fragmented filters was added into Lysing Matrix E tubes along with sodium phosphate buffer and MT buffer. All the E tubes were then homogenized vigorously on the FastPrep instrument (MP Biomedicals, Solon, OH, USA) to break up microbial cells and release nucleic acid. Subsequent extraction and purification steps were also performed in accordance with the manufacturer's instructions. Finally, genomic DNA was eluted in 100 μL of DNase/Pyrogen-free water and DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Inhibitors, such as polyphenols, humic acid, and tannic acid, were removed using a One Step™ PCR Inhibitor Removal Kit (Zymo Research Corps, Irvine, CA, USA). The concentration and purity of genomic DNA were determined again using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted genomic DNA was stored at –20°C until further analysis.

2.3 PCR assays

Three MST assays based on *Bacteroidales* 16S rRNA gene markers, and previously used in relation to pig waste (PF, Pig-Bac1^{SYBR}, and Pig-Bac2^{SYBR}) were performed in order to qualitatively evaluate their performance by PCR. All pig-specific primers used in this study are shown in Table 1. The 25 μL PCR mixture contained 5 μL of 10 × Ex Taq buffer (Mg^{2+} plus) (TaKaRa Corporation, Dalian, China), 4 μL dNTP Mixture (2.5 mM of each), 0.25 μL TaKaRa Ex Taq (5 U/μL), and 1 μL BSA (5 mg/mL). One microliter of forward and reverse primers (25 mol/L), 1 μL of genomic DNA template, and sterile ultrapure water were added to reach the final volume. Reagent preparation was carried out on a clean bench equipped with a UV-light for sterilization before and after use. A negative control was set up to determine whether the reagents were contaminated during the experiment. The PCR reaction for the PF assay was subjected to a pre-denaturation step at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 53°C–65°C (with a 1°C gradient) for 1 min, 72°C for 30 s, and finally at 72°C for 10 min. The specificity of the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays, which are based on SYBR green qPCR (Okabe et al., 2007), were first evaluated using normal PCR. Following Okabe et al. (2007), the reaction was subjected to pre-denaturation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min (Table 1). All the PCR products were electrophoresed on a 1% agarose gel with DNA Marker DL2000 (TaKaRa Corporation, Dalian, China).

Table 1 Sequences of primers and probes used for pig-specific *Bacteroidales* assays

Assay	Primers or probes	Sequence (5′– 3′)	Final concentration	Annealing temperature (°C)	Amplicon size (bp)
PF (Bernhard and Field, 2000; Dick et al., 2005)	PF163F	GCGGATTAATACCGTATGA	1 mol/L	62	563
	Bac708R	CAATCGGAGTTCTTCGTG	1 mol/L		
Pig-Bac1^{SYBR} (Okabe et al., 2007)	PS422F	CGGGTTGTAAACTGCTTTTATGAAG	1 mol/L	62	150
	Bac581R	CGCTCCCTTTAAACCCAATAAA	1 mol/L		
Pig-Bac2^{SYBR} (Okabe et al., 2007)	Bac41F	TACAGGCTTAACACATGCAAGTCG	1 mol/L	62	150
	PS183R	CTCATACGGTATTAATCCGCCTTT	1 mol/L		
Pig-1-Bac^{TaqMan} (Mieszkin et al., 2009)	Bac32F	AACGCTAGCTACAGGCTTAAC	0.2 mol/L	60	129
	Bac108R	CGGGCTATTCCTGACTATGGG	0.2 mol/L		
	Bac44p	(FAM)ATCGAAGCTTGCTTTGATA-GATGGCG(BHQ-1)	0.2 mol/L		
Pig-2-Bac^{TaqMan} (Mieszkin et al., 2009)	Bac41F2	GCATGAATTTAGCTTGCTAAATTTGAT	0.3 mol/L	60	116
	Bac163R	ACCTCATACGGTATTAATCCGC	0.3 mol/L		
	Bac113p	(VIC)TCCACGGGATAGCC(NFQ-MGB)	0.2 mol/L		

2.4 qPCR assays

2.4.1 Experimental process

According to Mieszkin et al. (2009), two probe-based assays, Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan}, can quantitatively identify the source of pig fecal pollution. In this study, we used the reaction conditions and reagent concentrations (primers and probes) specified in the original study. Standard plasmids for qPCR were constructed by Sangon Biotech (Shanghai) Co., Ltd. The standard curve was plotted using the quantitative results of a serial dilution (10^8–10^2) of each solution of plasmid containing a pig-specific marker. The 20 μL qPCR mixture contained 10 μL of Premix Ex Taq (Probe qPCR) (TaKaRa Corporation, Dalian, China), 0.4 μL of ROX reference dye (50 ×), 0.4 μL of BSA (5 mg/mL), 0.5 μL of genomic DNA template, and primers and probes, both of which were added based on their final concentration in the mix (Table 1). Sterile ultrapure water was added to reach the final volume. All reagent preparation was carried out on a clean bench equipped with a UV-light for sterilization before and after use. Each 96-well plate was set up with three negative controls to determine whether the reagent was contaminated during the experiment. All the qPCR assays were performed on an ABI PRISM® 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The reaction was subjected to a pre-denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were subjected to three parallel analyses on separate 96-well plates. The results of qPCR assays were processed using SDS System Software v.1.4.0 (Applied Biosystems, Carlsbad, CA, USA) for calculating Ct values and gene marker concentrations, which were normalized to the $\log_{10}$ Gene Copies (GC) /g value of wet feces and the $\log_{10}$ GC/mL value of water. The differences in Ct between parallel analyses were examined to verify consistency (i.e. the difference of Ct value among parallel analyses was$\leqslant$1) (Nshimyimana et al., 2017). To minimize error, the same pipettes were used throughout the experiment. Reproducibility of qPCR assays on each 96-well plate was verified by using two standard dilutions of each plasmid (10^3 and 10^2 GC/μL DNA) to test together with other samples (Nshimyimana et al., 2017). Overall, the average coefficient of variation (%CV) of Ct values was 1.25±0.22% for the 10^2 GC/μL plasmids and 1.88±0.39% for the 10^3 GC/μL plasmids.

2.4.2 Limit of detection

According to United States Environmental Protection Agency (US EPA), the limit of detection (LOD) is the minimum concentration of a substance reported with 99% confidence, which is distinguishable from blank results (US EPA, 2016). Samples with a detected marker concentration below LOD are considered negative. The procedure for determining LOD according to the US EPA was as follows: 1) The initial LOD, which is the average concentration plus three times the standard deviation of a set of blanks, was estimated. 2) The spiking level was selected. This was eight times the initial estimated LOD, and we processed nine spiked samples and nine blank samples through the method. 3) The LOD was calculated, as follows:

$$\mathrm{LOD} = S \times t_{(n-1,1-a=0.99)} \quad (1)$$

In Eq. (1), "LOD" is the limit of detection; "$t_{(n-1,1-a=0.99)}$" is the Student's t-value, suitable for a single-tailed 99th percentile t-test with n–1 degrees of freedom; "n" is the number of samples; "S" is the standard deviation of the replicate spiked sample analyses.

2.5 Evaluation of assays' performance

The performance of each assay was evaluated in terms of sensitivity, specificity, and accuracy. These three indexes were derived from the tested results of all fecal samples using the following three equations (Kildare et al., 2007; Odagiri et al., 2015; Nshimyimana et al., 2017).

$$\mathrm{Sensitivity} = \mathrm{TP}/(\mathrm{TP} + \mathrm{FN}) \quad (2)$$

$$\mathrm{Specificity} = \mathrm{TN}/(\mathrm{TN} + \mathrm{FP}) \quad (3)$$

$$\mathrm{Accuracy} = (\mathrm{TP} + \mathrm{TN})/(\mathrm{TP} + \mathrm{FP} + \mathrm{TN} + \mathrm{FN}) \quad (4)$$

TP (true positive) is the number of target samples that tested positive (Eq. (2) and (4)); FN (false negative) is the number of target samples that tested negative (Eq. (2) and (4)); TN (true negative) is the number of non-target samples that tested negative (Eq. (3) and (4)); FP (false positive) is the number of non-target samples that tested positive (Eq. (3) and (4)).

2.6 Statistical analysis

The significance of differences between the results of quantitative MST assays (Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan}) was determined by t-test using R version 3.4.3. This compared the concentrations of gene markers in target samples detected by these two assays to select a suitable assay for MST of pig fecal pollution.

3 Results

3.1 Qualitative assays

We used the pig-specific markers PF, Pig-Bac1 and Pig-Bac2 to qualitatively analyze pig, cow, goat, horse, and

human feces. Initially, we evaluated the performance of the PF assay, at an annealing temperature of 53°C. Following agar gel electrophoresis of amplicons, we observed target bands representing 16S rRNA gene markers (563 bp) in all pig fecal samples, in 83% of human fecal samples, and in all horse fecal samples. Meanwhile, there were many faint non-target bands in fecal samples of pigs (80%), cows (67%), goats (67%), and humans (83%). We therefore raised the annealing temperature in a gradient (1°C) from 53°C to 65°C to improve specificity. This revealed that the PF assay performed best at an annealing temperature of 62°C. Target bands were only detected in pig fecal samples. The optimized PF assay had 100% specificity, sensitivity, and accuracy (Table 2).

For the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays, normal PCR was initially performed under the reaction conditions recommended by Okabe et al. (2007). Agar gel electrophoresis of the amplicons from the two assays showed the target bands (150 bp) in almost all fecal samples (Table 2). The Pig-Bac1 marker was detected in 96% of pig samples, 83% of cow samples, 56% of goat samples, 52% of chicken samples, 80% of human samples, and 100% of horse samples, showing a specificity of 35% (Table 2). Similar results were found for the Pig-Bac2^{SYBR} assay, with the target band detected in 100% of pig fecal samples, 96% of cow fecal samples, 64% of goat fecal samples, 86% of chicken fecal samples, 95% of human fecal samples, and 100% of horse fecal samples (specificity 15%; Table 2). In addition, faint non-target bands were detected in many samples by these two assays.

3.2 Quantitative assays

The two pig-specific gene makers Pig-1-Bac and Pig-2-Bac gave positive signals in all pig fecal samples (n = 80) at high concentrations by TaqMan qPCR. The LODs of the Pig-1-Bac and Pig-2-Bac assays were 17 GC/μL DNA and 2310 GC/μL DNA, respectively. Overall, these assays showed 100% specificity, sensitivity, and accuracy (Table 2). The average concentration of Pig-1-Bac and Pig-2-Bac in pig wet feces was 7.95±1.55 $\log_{10}$ GC/g and 9.20±1.03 $\log_{10}$ GC/g wet feces, respectively (Table 3 and Fig. 2). In contrast, signals of the two markers in non-pig fecal samples were all below the calculated LOD, allowing pig fecal samples to be quantitatively distinguished from other non-target fecal samples. Concentrations of the markers detected by these two quantitative assays in pig fecal samples were not significantly different by t-test (P = 0.074; Table 3). Therefore, both assays can be used to quantitatively detect pig fecal contamination. However, the Pig-2-Bac^{TaqMan} assay showed better data reproducibility than the Pig-1-Bac^{TaqMan} assay (Fig. 2), as indicated by its lower coefficient of variation (Table 3). We therefore selected the Pig-2-Bac^{TaqMan} assay for the field test.

3.3 Field test

Based on the above performance validation, we selected the PF and Pig-2-Bac^{TaqMan} assays for qualitatively and quantitatively determining pig fecal pollution in two rivers near a pig farm (Fig. 1). The sampling was carried out twice at each site, with an interval of about half a year, during which the local environmental protection agency took measures to control the pig fecal pollution. As shown in Table 4, target bands were detected in the samples from six of the seven sites (H-1, H-2, H-3, H-4, H-5, and J-7) before pollution governance, with the strongest target band appearing at H-5. Correspondingly, the quantitative Pig-2-Bac^{TaqMan} assay also detected positive signals in the samples from the above mentioned six sites, with the

Table 2 Performance of pig-specific *Bacteroidales* assays for detecting fecal samples

Assay	Number of samples showing positive results						Specificity	Sensitivity	Accuracy
	Organism of origin								
	Pigs (n = 80)	Cows (n = 23)	Goats (n = 25)	Chickens (n = 21)	Humans (n = 20)	Horses (n = 4)			
PF	80	0	0	0	0	0	100%	100%	100%
Pig-Bac1	77	19	14	11	16	4	35%	96%	61%
Pig-Bac2	80	22	16	18	19	4	15%	100%	54%
Pig-1-Bac	80	0	0	0	0	0	100%	100%	100%
Pig-2-Bac	80	0	0	0	0	0	100%	100%	100%

Table 3 Quantitative comparison of Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays for analyzing pig fecal samples

Assays	C_0[a] ($\log_{10}$ GC/g wet feces)	Mean C_t[b]	%CV[c]
Pig-1-Bac	7.95±1.55	20.60	21.30
Pig-2-Bac	9.20±1.03	20.73	17.94
P value of t-test	0.074 (>0.05)	–	–

Notes: [a]Values reported are the mean and standard deviation of the log-transformed values of marker concentrations in the target and non-target samples; [b]Mean C_t value obtained from four plates; [c]Coefficient of variation of C_t values.

exception of the samples from J-6 (Fig. 3). The Pig-2-Bac concentration in the H-5 samples reached a maximum of about 3.73 $\log_{10}$ GC/mL water (Fig. 3). After the environmental governance, no target PF band was detected in any water sample, and the Pig-2-Bac concentration in all water samples was below the calculated LOD.

4 Discussion

Studies have reported that microorganisms undergo genetic variation while adapting to the gut environment of specific animal hosts (Shanks et al., 2010), and this may lead to region-specific performance differences among MST assays based on host-specific gene markers. Therefore, the performance of MST assays needs to be validated prior to field testing in order to confirm their specificity, sensitivity, and accuracy in the relevant context.

4.1 Performance validation

The PF assay was originally developed by Bernhard and Field (2000) and Dick et al. (2005). Although the PF marker has been detected with 100% sensitivity in pig fecal samples from many regions (Gourmelon et al., 2007; Malla et al., 2018; Somnark et al., 2018a), cross-reactions have sometimes been found with fecal samples from chickens (Gourmelon et al., 2007; Malla et al., 2018; Somnark et al., 2018b), cows, and goats (Somnark et al., 2018a; Somnark et al., 2018b). In this study, the PF assay mainly cross-reacted with human and horse fecal samples annealing at 53°C, but it achieved better performance after optimization at an annealing temperature of 62°C, with which it gave 100% specificity, sensitivity, and accuracy (Table 2).

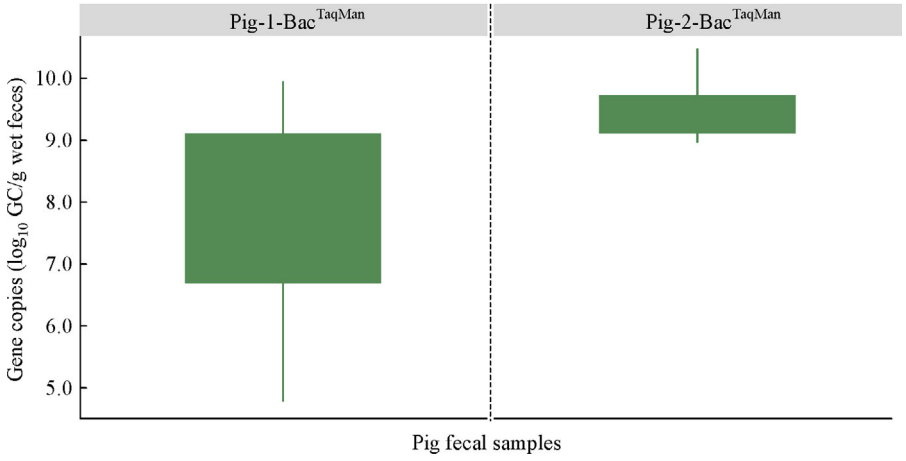

Fig. 2 Boxplot representation of pig-specific marker concentration in fecal samples detected by Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays.

Table 4 Qualitative PF assay for environmental water samples before environmental governance.

PF assay$_{(n=3)}$	Sampling sites						
	H-1	H-2	H-3	H-4	H-5	J-6	J-7
Number of positive samples	2	3	1	3	3	0	3
Intensity of target band	++	++	++	++	+++	−	+

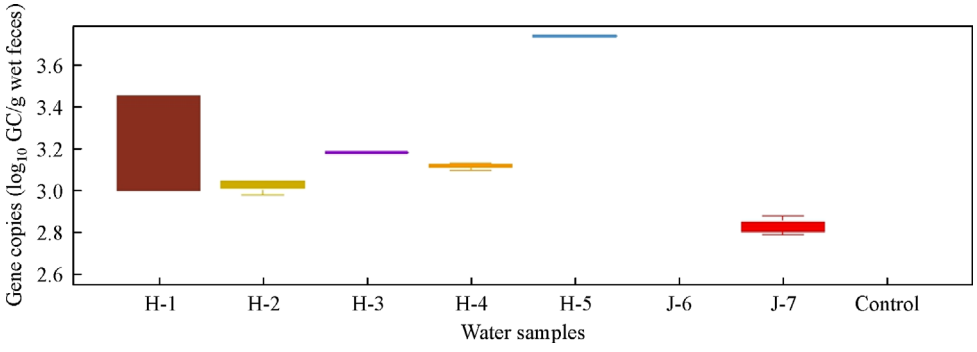

Fig. 3 Boxplot representation of concentration of the marker Pig-2-Bac in environmental water samples before environmental governance.

Similar results were observed in Canada (Fremaux et al., 2009), where the PF assay showed 100% sensitivity, specificity, and accuracy for determining pig fecal pollution at an annealing temperature of 57.4°C. Based on our results, with the increase of annealing temperature, disappearance of the target band in non-pig fecal samples indicates that the PF primers can mismatch with genomes of non-pig samples under non-optimized conditions.

A study in Japan reported that the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays could quantitatively detect makers in pig fecal samples in the order of 9–11 $\log_{10}$ GC/g wet feces (Okabe et al., 2007). Given that SYBR green qPCR is susceptible to interference from non-target amplification and may produce false-positive results, we here used agarose gel electrophoresis to detect the PCR products of the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays. However, target bands were found in almost all fecal samples from pigs, cows, goats, chickens, horses, and humans. Cross-reactions with non-target samples from chickens and cows were also found by Wang et al. (2014) in the Pearl River Delta region of China. In addition, the results of a study in France (Mieszkin et al., 2009) showed that the Pig-Bac2^{SYBR} assay produced positive signals in non-target samples from humans, cows, goats, and horses, showing specificity of 54%. Consistent with these studies, our results indicate that the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays are not suitable for source tracking of pig fecal pollution in China.

In contrast to the Pig-Bac1^{SYBR} and Pig-Bac2^{SYBR} assays, the Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays are widely used in various regions. According to the assays' developers, Mieszkin et al. (2009), they are capable of high sensitivity (98% and 100%, respectively) and specificity (both 100%) for quantitatively detecting pig fecal pollution in Brittany (France). However, there are a few reports on the potential of these assays for cross-reactivity. For example, a study in Canada found that the Pig-1-Bac^{TaqMan} assay cross-reacted with chicken fecal samples, though it still showed a high specificity of 95.7% (Ridley et al., 2014). Other studies have shown that the Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays have consistently high sensitivity and specificity (>90%) (Ridley et al., 2014; Heaney et al., 2015), indicating that they might not be significantly geographically constrained. In this study, both assays showed 100% sensitivity and specificity. Notably, the Pig-2-Bac^{TaqMan} assay was the more reproducible of two (Fig. 2 and Table 3).

4.2 Field test

Both the results of the qualitative PF assay and the quantitative Pig-2-Bac^{TaqMan} assay revealed six sites (H-1, H-2, H-3, H-4, H-5, and J-7) contaminated by pig feces before pollution governance (Table 4 and Fig. 3) in these two rivers. Pig fecal pollution detected at sites H-1 and H-2 upstream of the pig farm suggests the presence of sources of pig feces other than the pig farm. The highest level of pig fecal pollution was at site H-5, which might be due to environmental conditions favorable for pollutant accumulation, such as low water level, slow flow rate, and evaporation of water. Downstream of the convergence of Rivers H and J, pig fecal pollution appeared at J-7, indicating that the River J has been contaminated by pig feces from River H (Table. 4 and Fig. 3). It's worth noting that not all samples from H-1 (1/3) and H-3 (2/3) gave positive results (Table 4 and Fig. 3), which may be due to uneven distribution of feces in water. Therefore, in order to accurately evaluate pig fecal pollution in water, repeat sampling is necessary. After environmental pollution governance, both our qualitative and quantitative assays detected no pig fecal pollution in all water samples, highlighting the potential effectiveness of the pollution governance.

Accordingly, both the PF and Pig-2-Bac^{TaqMan} assays can be employed to identify pig fecal pollution in these two rivers of North China. The PF assay is more suitable for quickly detecting the presence of pig fecal pollution in the field due to its ease of operation and low cost, while the Pig-2-Bac^{TaqMan} assay can quantitatively determine the level of pig fecal pollution. Overall, the PF and Pig-2-Bac^{TaqMan} assays validated in this study could help the environmental protection agency to effectively supervise pig fecal pollution in rivers.

5 Conclusions

In this study, we validated the performance of five pig-specific MST assays employing *Bacteroidales* 16S rRNA as a genetic marker.

- The optimized PF assay showed 100% specificity, sensitivity, and accuracy, and has practical advantages for qualitatively determining pig fecal pollution.
- The quantitative Pig-1-Bac^{TaqMan} and Pig-2-Bac^{TaqMan} assays also showed 100% sensitivity, specificity, and accuracy, while the latter had better reproducibility.
- The field test demonstrated that the application of the pig-specific MST assays (PF and Pig-2-Bac^{TaqMan}) can contribute to the management of pig fecal pollution in these two rivers, and therefore we recommend these two assays for application elsewhere in China.

Acknowledgements This work was supported by the National Key Research and Development Program of China (No.2016YFC0503601), and Excellent Young Teachers' Scientific Research Ability Improvement Project of University of Chinese Academy of Sciences (No. Y95401FXX2).

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