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Research article

Evaluation of *Faecalibacterium* 16S rDNA genetic markers for accurate identification of swine faecal waste by quantitative PCR

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ABSTRACT

A genetic marker within the 16S rRNA gene of *Faecalibacterium* was identified for use in a quantitative PCR (qPCR) assay to detect swine faecal contamination in water. A total of 146,038 bacterial sequences were obtained using 454 pyrosequencing. By comparative bioinformatics analysis of *Faecalibacterium* sequences with those of numerous swine and other animal species, swine-specific *Faecalibacterium* 16S rRNA gene sequences were identified and Polymerase Chain Okabe (PCR) primer sets designed and tested against faecal DNA samples from swine and non-swine sources. Two PCR primer sets, PFB-1 and PFB-2, showed the highest specificity to swine faecal waste and had no cross-reaction with other animal samples. PFB-1 and PFB-2 amplified 16S rRNA gene sequences from 50 samples of swine with positive ratios of 86 and 90%, respectively. We compared swine-specific *Faecalibacterium* qPCR assays for the purpose of quantifying the newly identified markers. The quantification limits (LOQs) of PFB-1 and PFB-2 markers in environmental water were 6.5 and 2.9 copies per 100 ml, respectively. Of the swine-associated assays tested, PFB-2 was more sensitive in detecting the swine faecal waste and quantifying the microbial load. Furthermore, the microbial abundance and diversity of the microbiomes of swine and other animal faeces were estimated using operational taxonomic units (OTUs). The species specificity was demonstrated for the microbial populations present in various animal faeces.

1. Introduction

Water contaminated with faeces has been a crucial issue in many countries (Araujo et al., 2014; Zhang et al., 2015; Kumar et al., 2016). The water quality of many waterways and coastal waters is deteriorating due to point and non-point sources of faecal contamination caused by both human and animal sources (Tran et al., 2015). Faecal contamination of water increases the risk of waterborne disease and poses potential health risks to humans (Haile et al., 1999). Thus, it is important to identify the sources of faecal contamination and determine the degree of faecal pollution. Once the major contamination source is identified, appropriate management and remediation efforts can proceed in a more timely and cost-effective manner (Johnston et al., 2013; Nnane, 2011).

Historically, faecal indicator bacteria (FIB) such as *Enterococcus* spp. and *Escherichia coli* were used as indicators of faecal pollution of fresh and marine waters in water utilisation (Dombek et al., 2000). However, several critical limitations have been described, including the inability to detect non-culturable bacteria, difficulty in identifying faecal sources (Field and Samadpour, 2007), poor correlation with the presence of enteric pathogens (Harwood et al., 2005), and lack of information about the host source(s) of contamination (Scott et al., 2002). Various microbial source tracking (MST) methods have been developed to address these limitations (Stapleton et al., 2009; Ahmed et al., 2014). Among a variety of proposed MST techniques, host-associated *Faecalibacterium* genetic markers are increasingly used as a complement or

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alternative to standard FIB in the developed world (Zheng et al., 2009; Shen et al., 2013). Within the phylum *Firmicutes*, *Faecalibacterium* is a newly established genus, which includes a single species of *Faecalibacterium prausnitzii* with the type strain reclassified as *Faecalibacterium prausnitzii* (ATCC27768) (Acinas et al., 2004). *Faecalibacterium* is a unique gram-negative, obligate anaerobe that is non-spore forming and non-motile. Furthermore, *F. prausnitzii* is regarded as one of the most prevalent bacteria of the resident microbiota within the human gastrointestinal tract (GIT) and is present in the faeces of humans and other animals (Walker et al., 2011). Studies have shown that *Faecalibacterium* plays an important role in the healthy gut (Lukovac et al., 2014; Lopez-Siles et al., 2015). Recent studies have demonstrated the relevance of MST using *Faecalibacterium* genetic markers to identify faecal sources, including human and poultry (Zheng et al., 2009; Shen et al., 2013). To our knowledge, however, no MST *Faecalibacterium* assays have been developed to address swine faecal pollution problems in the world.

Suppression subtractive hybridisation (SSH) and cloning were used to identify genetic markers (Clermont et al., 2008; Boehm et al., 2013). With the development of new technologies, 454 pyrosequencing has become a more powerful tool that can be used to analyse millions of nucleic acid sequences and generate a vast amount of data in a shorter time with a low level of error (0.1%) (Ratti et al., 2015). Sequence analysis of their 16S rRNA gene has enabled researchers to characterise previously unknown isolates and increase the current understanding of their distribution in the intestinal flora of different animals (Hold et al., 2002; Vaughan et al., 2015). Surprisingly little attention has been paid to the use of 454 pyrosequencing to analyse the microbiota in animal faeces.

It was estimated that the majority of water contamination by animal manure comes from swine, cattle, and poultry (Bohm, 2000). Several PCR- and qPCR-based MST systems have been used for the identification and quantification of water contamination (Jyoti et al., 2010; Odagiri et al., 2015; Validation et al., 2015), and most of them are based on 16S rRNA sequences (Field and Samadpour, 2007; Kobayashi et al., 2013). The scaling-up of the swine farming industry has resulted in serious water environmental problems from the large quantity of faecal waste (Heaney et al., 2015). Host-specific qPCR primers targeting 16S rRNA genes of *Bacteroides* and *E. coli* have been successfully developed to quantify genetic markers in water environments and to identify faecal sources from humans, cows or poultry (Reischer et al., 2007; Seurinck et al., 2005). However, qPCR assays targeting 16S rRNA genes of swine-associated *Faecalibacterium* have not yet been developed.

The objectives of this study were to: (i) estimate the abundance and diversity of the faeces microbiome by analysis the 16S rRNA gene sequences obtained using 454 pyrosequencing, (ii) identify swine-specific *Faecalibacterium* 16S rDNA sequence, (iii) develop PCR and qPCR methods for the evaluation of swine-associated markers, (iv) quantitatively measure the sensitivity and specificity of the swine markers and (v) apply the newly developed assay to an MST field study.

2. Materials and methods

2.1. Sample collection and DNA extraction

Faecal samples were collected with sterile utensils and placed in sterile 50-ml tubes. Human faecal samples (n = 18) were donated by a variety of individuals of varying age, sex, and ethnic backgrounds. Fresh faecal samples from swine (n = 31), cows (n = 22), chickens (n = 18), ducks (n = 10), and dogs (n = 8) were collected from various farms in Chongqing, China (Table 1). In addition, 19 individual swine faecal samples were collected from Jiaozuo, China. All samples were transported to the laboratory on ice and immediately stored at −80 °C before the DNA extraction was performed.

Environmental water samples were collected from various cities in sterile 250-ml bottles and transported on ice to the laboratory. River water samples were collected from Jialing River in Chongqing, Han River in Hanzhong and Wenxian River in Jiaozuo, China. Pond water samples were collected from Beibei, Chongqing. Seawater samples were collected from Yantai (Fig. 1). Sample bottles were coded so that sample processors were blinded during laboratory analysis. After the obvious impurities in environmental, samples were removed from the bottle with a sterile utensil, and filtered simultaneously onto 47-mm, 0.22 mM pore Supor-200® (Pall, Port Washington, NY, USA) filters using a filtration manifold. Bacteria on membrane filters were eluted by soaking in 30 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.6) and stored for 8 h at 4 °C, then rigorously shaken by a vortex mixer. Suspensions in the STE buffer were precipitated by centrifugation at 10,000 g for 20 min at 4 °C. After the supernatant was gently removed, the washed cells were resuspended in 2-ml centrifuge tubes containing 200 μl of distilled MilliQ water for washing and subjected to DNA extraction (Green et al., 2011).

Genomic DNA from faecal samples (0.25 g) and concentrated water samples (200 μl) was extracted using the PowerSoil® DNA extraction kit (MoBio Laboratories, Carlsbad, CA) as previously described (Zheng et al., 2009). DNA was eluted in 100 μl of elution buffer and stored at −20 °C before use.

2.2. 454 pyrosequencing

The faecal microbiota from the chicken, duck, human, swine and cow were characterised in samples subjected to 16S rRNA gene analysis via 454 sequencing on a Roche 454 Titanium platform (454 Life Sciences, Branford, CT). We performed multiplex pyrosequencing with a 454 FLX instrument to survey the gene's V3 and V6 variable regions. The obtained FASTA files were uploaded in the Ribosomal Database Project (RDP) pipeline initial processor that trimmed the 16S primers and filtered out additional sequences of low-quality (Wright et al., 2012). The RDP Classifier at the RDP's Pyrosequencing Pipeline was used to assign 16S rRNA gene sequences of each sample to the new phylogenetically consistent higher-order bacterial taxonomy (Wang et al., 2007). For subsequent processing of high-throughput data, we grouped 16S rRNA gene sequences into operational taxonomic units (OTUs) using mothur v1.30.0, with a sequence identity threshold of 97%, which is commonly used to define 'species'-level phylotypes (Schloss, 2009). The sequences were then classified into phylotypes using the RDP public 16S rDNA database (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Oikonomou et al., 2013). Based on the classification of the microbiota, we analysed the microbiota in chicken, duck, human, dog, swine and cow faecal samples. Finally, alpha diversity for each sample was established using QIIME with default parameters. The Simpson index and of Shannon-wiener index 16S rRNA gene OTUs were calculated according to the equations (1) and (2). The estimated alpha diversities were shown in Table 2

$$\text{D Simpson} = 1 - \sum (pi)^2 \quad (1)$$

$$\text{H Shannon-wiener} = -\sum pi \ln pi \quad (2)$$

where *pi* is the proportion of important value of the species (pi = ni/N), ni is the important value index of species and N is the important value index of all the species.