Environmental Pollution xxx (2017) 1–9

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Validation and application of quantitative PCR assays using host-specific *Bacteroidales* genetic markers for swine fecal pollution tracking☆

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ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form
14 September 2017
Accepted 15 September 2017
Available online xxx

Keywords:
Genome fragment enrichment
Bacterium-host interactions
Genetic markers
Real-time PCR assay
Microbial source tracking

ABSTRACT

Genome fragment enrichment (GFE) method was applied to identify host-specific bacterial genetic markers that differ among different fecal metagenomes. To enrich for swine-specific DNA fragments, swine fecal DNA composite (n = 34) was challenged against a DNA composite consisting of cow, human, goat, sheep, chicken, duck and goose fecal DNA extracts (n = 83). Bioinformatic analyses of 384 non-redundant swine enriched metagenomic sequences indicated a preponderance of *Bacteroidales*-like regions predicted to encode metabolism-associated, cellular processes and information storage and processing. After challenged against fecal DNA extracted from different animal sources, four sequences from the clone libraries targeting two *Bacteroidales*- (genes 1–38 and 3–53), a *Clostridia*- (gene 2–109) as well as a *Bacilli*-like sequence (gene 2–95), respectively, showed high specificity to swine feces based on PCR analysis. Host-specificity and host-sensitivity analysis confirmed that oligonucleotide primers and probes capable of annealing to select *Bacteroidales*-like sequences (1–38 and 3–53) exhibited high specificity (>90%) in quantitative PCR assays with 71 fecal DNAs from non-target animal sources. The two assays also demonstrated broad distributions of corresponding genetic markers (>94% positive) among 72 swine feces. After evaluation with environmental water samples from different areas, swine-targeted assays based on two *Bacteroidales*-like GFE sequences appear to be suitable quantitative tracing tools for swine fecal pollution.

1. Introduction

The presence of fecal contamination from animals in natural waters, fish harvesting and drinking waters may pose significant threat to public health (Fu et al., 2011; Soller et al., 2010). Pig farming has significantly expanded in China in the past few decades. Consequently, the high volume of swine fecal waste leads to a rising risk of the spread of enteric pathogens and environmental concerns such as high nutrient loads of water in China (Harwood et al., 2013; Lu et al., 2007). As reported, swine fecal contamination regarded as the carrier of zoonotic bacteria and parasites may lead to waterborne disease outbreaks, which were documented worldwide (Abdelzaher et al., 2010; Lapworth et al., 2012). Therefore, monitoring the source of fecal pollution is essential for the accurate evaluation of public health risks and emerging zoonotic infectious diseases (Ahmed et al., 2013; Lu et al., 2013).

Numbers of fecal source tracking methods have been developed over the last few decades to address the issue of swine-specific contributions of fecal contamination (Harwood et al., 2013; Hundesa et al., 2009; Ufnar et al., 2007; Khatib et al., 2003; Mieszkin et al., 2009; Okabe et al., 2007; Villemur et al., 2015). Among them, most specific markers targeting 16S rRNA gene of *Bacteroidales* spp. which constitutes a large proportion of the normal gut microbiota of most animals are commonly used to identify and quantify fecal pollution from swine (McLellan and Eren, 2014; Mieszkin et al., 2009; Okabe et al., 2007). Several methods based on toxin genes STII (Khatib et al., 2003), methanogen-specific mcrA genes (Ufnar et al., 2007) and porcine

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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https://doi.org/10.1016/j.envpol.2017.09.047
0269-7491/

adenovirus (PAdV) (Hundesa et al., 2009) were also developed for identifying swine waste. Although, several studies have demonstrated the value of swine specific gene-based assays, numbers of methods were found to lack of specificity and sensitivity in field samples (Mieszkin et al., 2009; Ufnar et al., 2007; Villemur et al., 2015). Alternative markers must be developed and tested to provide a more comprehensive complement for fecal source tracking. Functional genes directly involved in host-microbial interactions such as microbial surface protein genes, cellular processes and metabolism associated genes are considered to be a potential pool of targets for host-specific genetic markers (Lu et al., 2007; Xu et al., 2003). Several studies have successfully developed PCR assays based on genes involved in host-microbial interactions and confirmed that the genetic markers were highly specific to human, cattle or chicken, indicating the feasibility of functional genes for fecal source tracking (Lu et al., 2007; McLellan and Eren, 2014; Shanks et al., 2006). However, to date, there is no such functional genes-based assay that can determine the presence of swine fecal contamination.

The current study aimed to identify swine-specific markers targeting genes potentially involved in host-microbial interactions from metagenomic DNA, using a competitive DNA hybridization approach named genome fragment enrichment (GFE). Quantitative PCR assays based on swine-specific sequences enriched after competitive DNA hybridization were then developed and assessed for tracking swine fecal pollution in natural samples.

2. Materials and methods

2.1. Samples and DNA extraction

Swine fecal samples (n = 34) and feces from non-target animals including cow (n = 20), human (n = 13), goat (n = 12), sheep (n = 6), chicken (n = 7), duck (n = 20), goose (n = 5) from different herds located in separate geographic areas in Southeastern China including provinces of Zhejiang, Jiangxi, Fujian and city of Shanghai were collected aseptically to select potential swine-specific genes by genome fragment enrichment (GFE) method. Individual fecal samples from swine (n = 64), cow (n = 13), goat (n = 10), sheep (n = 8), chicken (n = 8), duck (n = 15), goose (n = 5), dog (n = 3) and human (n = 9), as well as piggery wastewater (n = 8), were collected for further test of host-specificity and sensitivity. Fecal samples were collected in sterile containers and approximately 500 mg (wet weight) of feces was mixed with 3 ml of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) and stored at −80 °C until used.

Natural water samples that were possibly contaminated by swine (n = 20), human (n = 3), cow (n = 4), duck (n = 2), chicken (n = 2) and goat (n = 9) were collected from different geographic regions in Southeastern China for evaluation of potential use of the genetic markers. Natural water samples were collected in sterile bottles and transported to the laboratory under the condition of 4 °C. One-hundred milliliter of each sample was filtered by 0.45 μm polycarbonate membranes (CN-6 Metricel® Grid 47 mm, life Science). Membranes were then placed in sterile conical tubes for DNA extraction immediately or stored at −80 °C until used.

All DNA extractions were performed with QIAamp® Fast DNA Stool Kit (Qiagen, Valencia, CA) following manufacturer's instructions. DNA concentrations were determined using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Thermo fisher).

2.2. GFE

Potential swine-specific metagenomic DNA fragments were enriched using GFE method described previously (Shanks et al., 2006) with some modifications. Briefly, matagenomic DNA extracts of 34 swine fecal samples were mixed as a fecal microbial community DNA composite, while DNA extracts of non-target animals including cow, human, goat, sheep, chicken, duck and goose were mixed to create a community DNA composite used as blocker. Biotin-labeled, sheared swine fecal DNA composite was prehybridized with sheared DNA fragments of blocker for 30 min. To prepare DNA used to enrich for host-specific fragments, K9-DNA primer (Grothues et al., 1993) were linked to sheared swine fecal DNA using Klenow I polymerase extension (New England BioLabs, Ipswich, MA). K9-labeled DNA fragments were then hybridized to equilibrium in solution with the "blocked" biotin-labeled DNA. Biotinylated DNA hybrids were isolated by streptavidin binding. After amplification of captured K9-tagged genomic fragments by lone-linker PCR (Grothues et al., 1993), the products were purified with QIAquick® Multiwell PCR Purification Kit (Qiagen, Valencia, CA) and used for the next round of enrichment. Finally, PCR products for each round were pooled and cloned into pCR TOPO 4.0 following the manufacturer's instructions (Invitrogen, Carlsbad, CA). All PCR reactions were performed on Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA).

2.3. Dot blot hybridization

To identify the host-specificity of swine sequences obtained by GFE, dot blot hybridizations with cloned sequences and a probe of fecal community DNA composite of other animals (GFE blocker) were performed as previously described (Shanks et al., 2006). Briefly, purified PCR products from enriched swine DNA sequences were denatured and spotted directly onto nylon membranes (Li-Cor Biosciences, Lincoln, NE). After UV cross-linked and blocked with salmon sperm DNA (Sigma-Aldrich), the spotted DNA were hybridized with a biotin-labeled fecal community DNA composite of non-target animals. Membranes were visualized with an FluoRChem HD2 Imaging System (ProteinSimple, USA), following detection with KeyGEN Super ECL Assay (KeyGEN BioTECH, China) catalyzed by the streptavidin-horseradish peroxidase (HRP) conjugate (Invitrogen, Carlsbad, CA). 1.0 μg of fecal composite DNA of non-target animals was spotted onto nylon membranes and served as positive control.

2.4. Sequencing and data analysis

Randomly selected recombinant *E. coli* clones containing inserts of GFE DNA fragments were sent to Shengong Ltd. (Beijing, China) for sequencing on an ABI PRISM 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

DNA sequence reads were edited and assembled using EditSeq and SeqMan II in DNAstar package (Madison, WI). Each sequence was analyzed using BLASTx in the non-redundant GenBank database (Altschul et al., 1997). Sequence with *E* values of $\leqq 10^{-3}$ and similarity of ≧30% were defined as homologous protein sequences (Breitbart et al., 2003; Lu et al., 2007). Bacterial class annotations of GFE sequences were assigned via the top BLASTx hit in the non-redundant GenBank database. Gene function attributes of enriched sequences were evaluated with the database of Clusters of Orthologous Groups of proteins (http://www.ncbi.nlm.nih.gov/COG).

Sequences of GFE genes were provided in the Supporting file.

2.5. Primers and probes

Primers targeting enriched DNA fragments were designed and

Please cite this article in press as: Fan, L., et al., Validation and application of quantitative PCR assays using host-specific *Bacteroidales* genetic markers for swine fecal pollution tracking, Environmental Pollution (2017), https://doi.org/10.1016/j.envpol.2017.09.047