Q-254 Significant Cross-Amplification of Fish Fecal DNA Using Quantitative Real-Time PCR Specific to the Human *Bacteroides* 16S rRNA Genetic Marker

Leila Kabiri-Badr[1], Hodon Ryu[1] , Jean E.T. McLain [2], Channah Rock[3], Absar Alum[1], Morteza Abbaszadegan[1]
[1]National Science Foundation Water Quality Center, Arizona State University, Tempe, AZ, [2]USDA-ARS, US Arid-Land Agricultural Research Center , Maricopa, AZ, [3]University of Arizona, Maricopa, AZ.

Contact Information:
Leila Kabiri
PO Box 875306, Tempe, AZ 85287
Tel: 480-965-7978
E-mail: leila.kabiri-badr@asu.edu

Introduction

- Increased use of recycled wastewater for municipal irrigation heightens the importance of accurate source tracking methods to differentiate sources of fecal contamination in environmental samples.
- *Bacteroides*-specific molecular markers and quantitative real-time PCR have been widely used to discriminate human fecal bacteria from other sources, but cross-reactivity of the human *Bacteroides* primers with canine and swine feces has been reported **(1).**
- The objective of this study was to investigate the cross-reactivity of a Taq man PCR assay specific to the human *Bacteroides* 16S rRNA genetic marker with known sources of fecal bacteria. The study site was in a municipal park in Maricopa, Arizona, where a pond filled with tertiary treated municipal wastewater and stocked for recreational fishing was monitored for levels of human *Bacteroides.* Potential for cross-amplification was checked using samples collected from potential fecal contaminants (e.g., dogs, birds, fish).
- Development of highly specific source tracking methods will allow accurate assessment of public health impacts resulting from the use of this pond water for municipal irrigation.

Materials and Methods

Field

Sample collection

Water Sample Collection

Grab samples of the pond water were collected from March to June 2007 and filtered through sterile 0.45 µm filters.

Fecal Sample Collection

Fresh fecal samples were collected from animals (dog, bird and fish) and humans and transported to the laboratory on ice.

Fecal Sample Processing

PBS was added to fecal samples in a 75:25 mixture. After vortexing, 1.0 mL aliquots were removed for DNA extraction.

Figure 1. Site of water sampling: Pond in Pacana Park

Figure 2. Fish feces collection: Tilapia

Laboratory

DNA Extraction and Purification

DNA of both fecal and water samples were extracted and purified using ZR Fecal DNA Kit™ (Zymo Research, Orange, CA).

Quantitative Real-Time PCR

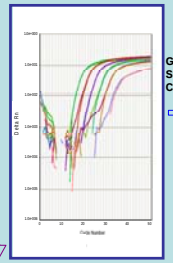
The probe-based Real-Time PCR assay developed by Layton,et al. **(1)** was used to quantify the total and human *Bacteroides* in the park water samples, using an ABI 7000 real-time PCR machine (Applied Biosystems, Foster City, CA).

Table 1. Oligonucleotide primers and probes for qPCR applied from Layton. et al (1).

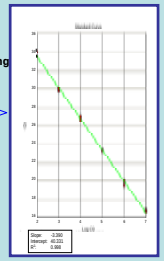
Assay	Primer/probe name and sequence (5'-3')*	Size(bp) of product	Annealing temp (°C)
AllBac (all *Bacteroides*)	AllBac296f, 5'-GAGAGGAAGGTCCCCCAC-3' AllBac412r, 5'-CGCTACTTGGCTGGTTCAG-3' AllBac375Bhqsr, 5'-(FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)-3'	100	60
HuBac (human cluster *Bacteroides*)	HuBac526f, 5'-GGGTTTAAAGGGAGCGTAGG-3' HuBac602r, 5'-CTACACCACGAATTCCGCCT-3' HuBac556Bhqf, 5'-(FAM)TAAGTCAGTTGTGAAAGTTTGCGGCTCG(BHQ-1)-3'	116	60

(+) Conformation Step

Direct Cloning and Sequencing



Generating Standard Curve



Standard PCR

Cloning inserts were developed using standard PCR.

Purification

PCR products were purified using QIAquick PCR Purification kit (Qiagen Sciences,Valencia, CA)

Cloning

PCR products were inserted to the linearized vector using a (TOPO TA cloning kits, Invitrogen Life Technologies, Carlsbad, CA)

Plasmid PCR

Colonies were screened for inserts from PCR with Plasmid-vector specific primers T3 and T7.

ExoSAP-IT (USB Corp., Cleveland, OH) was used for removal of unincorporated primers

DNA sequencing for insert DNA of interest

DNA Sequencing

Confirmed Plasmid inserts were directly sequenced using an automated ABI Prism 377 DNA sequencer with a Big Dye sequencing kit (Applied Biosystems, Foster City, CA)

Data Analysis

- Blast Program
- ClusterX software

Results

Table 2. *Bacteroides* in Pacana Park Water, Spring 2007

Sampling Date	Probe Targets 100 mL^{-1} All Bac	Hu Bac	HuBac % of Total
March 01	3.0 x 10^6	2.8 x 10^6	93%
March 29	1.5 x 10^7	1.6 x 10^7	106%
April 26	8.7 x 10^6	7.1 x 10^6	82%
May 27	4.5 x 10^5	4.3 x 10^5	95%
June 20	1.5 x 10^6	1.8 x 10^6	120%

High levels of fecal bacteria

Majority of the fecal contamination possibly of human origin

Results

Quantitative Real-Time PCR

- Using quantitative Real-Time PCR, high levels of fecal bacteria were detected in the pond (Table 2).
- *Bacteroides*-based source tracking using Q-PCR indicated that the majority of fecal contamination in the pond water was of human origin (Table 2).

Human vs. Fish: *Cloning and DNA Sequencing*

- The human-specific *Bacteroides* primers and probe were further investigated for their cross-reactivity against known sources of fecal contamination, and significant cross-amplification with fish (Tilapia spp.) feces was noted.
- To further investigate the cross reactivity between human *Bacteroides* and fish *Bacteroides,* cloning and DNA sequencing was performed.

Sequencing results of HuBac Amplicons Generated from Human (top) and Fish (bottom) Feces

Summary

- To our knowledge, no studies to date have examined cross-amplification of a human *Bacteroides* assay with fish feces.
- All clones (human and fish) showed 100% homology to the Q-PCR primer and probe sequences.
- The nucleotide sequences from all human clones were identical; however, mismatches were identified between the fish and human PCR products, indicating that the fish fecal bacteria were unique strains of *Bacteroides*, and that observed cross-reactivity was not the result of contamination of the fish fecal samples with human feces.

Further Study

- Analysis of *Bacteroides* from fish species other than tilapia (catfish, trout) is in progress
- Designing new set of primers and probe for human specific *Bacteroides*.

Literature Cited

1) Layton, et al. (2006) Development of *Bacteroides* 16S rRNA gene TaqMan-based Real-Time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72:4214-4224

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