Systematic and Applied Microbiology 33 (2010) 348–357

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected *Bacteroidales* species and human fecal waste by qPCR

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ARTICLE INFO

Article history:
Received 22 January 2010

Keywords:
QPCR
Bacteroidales
Species
Human
Fecal
Sources

ABSTRACT

Molecular methods for quantifying defined *Bacteroidales* species from the human gastrointestinal tract may have important clinical and environmental applications, ranging from diagnosis of infections to fecal source tracking in surface waters. In this study, sequences from the V2 region of the small subunit ribosomal RNA gene were targeted in the development of qPCR assays to quantify DNA from six *Bacteroides* and one *Prevotella* species. *In silico* and experimental analyses suggested that each of the assays was highly discriminatory in detecting DNA from the intended species. Analytical sensitivity, precision and ranges of quantification were demonstrated for each assay by coefficients of variation of less than 2% for cycle threshold measurements over a range from 10 to 4×10^4 target sequence copies. The assays were applied to assess the occurrence and relative abundance of their target sequences in feces from humans and five animal groups as well as in 14 sewage samples from 13 different treatment facilities. Sequences from each of the species were detected at high levels (>10^3 copies/ng total extracted DNA) in human wastes. Sequences were also detected by each assay in all sewage samples and, with exception of the *Prevotella* sequences, showed highly correlated ($R^2 \geq 0.7$) variations in concentrations between samples. In contrast, the occurrence and relative abundance profiles of these sequences differed substantially in the fecal samples from each of the animal groups. These results suggest that analyses for multiple individual *Bacteroidales* species may be useful in identifying human fecal pollution in environmental waters.

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Introduction

PCR-based methods have been developed to detect genetic markers from fecal indicator bacteria for the rapid determination of microbiological water quality [4,19,29]. This approach has been particularly useful for the rapid prediction of health risks associated with exposures to fecal polluted surface waters during recreational activities [43,44]. The determination of relative contributions by different animal sources to the total fecal pollution load in impaired waters in order to implement rational strategies for remediation is another potential application for these types of methods [33].

Members of the *Bacteriodales* taxonomic order have received much attention as genetic targets for molecular methods due to their high abundance in feces, as well as their low potential for regrowth in the environment. Several PCR-based assays for detecting and/or quantifying this entire order of bacteria have been reported and applied to the analysis of surface waters [13,21,26,39]. PCR assays targeting these organisms have also been at the forefront of efforts to develop methods for the detection of fecal pollution from specific animal hosts. An early prototype of this approach to fecal source tracking (FST) was a report on conventional PCR assays specifically directed at small subunit ribosomal RNA (ssrRNA) genetic markers from several known *Bacteriodes* species with human associations [24].

A second, more widely known approach is the use of conventional PCR assays targeting the HF134 and HF183 ssrRNA genetic markers from unknown, human-associated *Bacteroides* sequence clusters and the CF128 and CF193 ssrRNA genetic markers from *Bacteriodales* species associated with ruminants [6,7]. The HF183 marker was subsequently used in a SYBR Green chemistry-based qPCR assay for quantitative assessments of human fecal pollution in surface waters [35]. Subsequent to these studies, numerous other conventional and qPCR assays targeting ssrRNA and other genes from defined and undefined assemblages within the *Bacteriodales* [9,12,21,26,30,36–38,41], as well as other fecal bacteria [22,48] have been developed for the purpose of detecting fecal pollution from human and other specific animal sources. Assays based on the HF183 genetic marker, however, continue to be among of the most

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0723-2020/$ – see front matter. Published by Elsevier GmbH.
doi:10.1016/j.syapm.2010.06.001

Table 1
qPCR assay primer and probe sequences.

Primer/probe (concentration)[a]	Target organism(s) or DNA	Sequence (5′–3′)	GenBank reference (base positions)	Reference
Forward primers				
HF183	*Bacteroides dorei*	ATCATGAGTTCACATGTCCG	AB242142 (180–199)	[7]
BfragF1 (2 μM)	*Bacteroides fragilis*	CATTATTAAAGGATTCCGGTAAAG	M11656 (209–231)	This study
BsteriF1 (4 μM)	*Bacteroides stercoris*	GGGATRATTATTAAAGAATTTCGGcTGT[b]	X83953 (174–201)	This study
BthetaF2	*Bacteroides thetaiotaomicron*	GACCGCATGGTCTTGTTATT	M58763 (201–220)	This study
BuniF2 (2 μM)	*Bacteroides uniformis*	CATAGTTCTTCCGCATGGTRGA	L16486 (186–207)	This study
BvulgF1 (2 μM)	*Bacteroides vulgatus*	CATCATGAGTCCRCATGTTCA	M58762 (189–209)	This study
PcoprF1 (3 μM)	*Prevotella copri*	CTCTAGAAGRCATCTGAARGAGATT	AB064923 (184–208)	This study
GenBacF3	Total *Bacteroidales*	GGGGTTCTGAGAGGAAGGT	M58763 (299–318)	[39]
Reverse primers				
BFDRev	Total *Bacteroidales*	CGTAGGAGTTTGGACCGTGT	M58763 (338–357)	[12]
Bac708R	Total *Bacteroidales*	CAATCGGAGTTCTTCGTG	M58763 714–731)	[7]
GenBacR4	Total *Bacteroidales*	CcGTCATCCTTCACGCTACT[b]	M58763 (409–428)	[39]
TaqMan® probes				
BFDFAM	Total *Bacteroidales*	CTGAGAGGAAGGTCCCCCACATTGGA	M58763 (306–331)	[12]
GenBacP2	Total *Bacteroidales*	CAATATTCCTCACTGCTGCCTCCCGTA	M58763 (354–380)	[39]
UC1P1	IAC plasmids	CCTGCCGTCTCGTGCTCCTCA		[40]; this study

[a] Primer and probe concentrations in the reaction mixes were 1 μM and 80 nM, respectively unless otherwise indicated.
[b] Deliberately introduced mismatched base with target sequence is denoted by lower case.

widely used approaches for characterizing human fecal pollution in the environment. Recent evidence for an association between the HF183 marker and the newly discovered species, *Bacteroides dorei* [3] provides additional support for the concept of species-specific targeting for FST applications. With the current availability of more rapid and quantitative analytical techniques, such as qPCR, assays targeting additional *Bacteroidales* species can now be readily and comprehensively investigated to determine the prevalence and abundance of the sequences that they detect in the fecal material of different animal sources.

The HF183 marker occurs in the V2 region of the ssrRNA gene. This hypervariable region has been reported to exhibit one of the highest degrees of sequence diversity in the ssrRNA gene among bacterial species making it a good candidate for the development of diagnostic assays [10]. In addition it is amplified by the widely used general primers for *Bacteroidales*, Bac32 and Bac708 [6], resulting in the availability of an extensive database of sequences in this region. In this study our first objective was to determine whether genetic marker sequences from the V2 region can be used to develop TaqMan® chemistry-based qPCR assays that are specific for the detection of several other selected *Bacteriodales* species or closely related species groups in addition to *B. dorei*. Our second objective was to make an initial assessment of the potential utility of the qPCR assays developed from these markers as tools for FST applications based on analyses of the relative abundance of their target sequences in fecal samples from human and several different animal sources, as well from a collection of municipal waste water samples.

Methods

Primers and probes

Primer and TaqMan® probe sequences and presumptive target organisms for each qPCR assay used in this study are shown in Table 1. The same reverse primer and probe sequences, BFDrev and BFDFAM [12], were used for all of the candidate species-specific qPCR assays. Reported microorganism species and GenBank sequence accession numbers of representative *Bacteriodales* ssrRNA gene sequences used to develop presumptive species-specific primers are listed in Table S1. Some of the sequences were from unreported taxonomic origins, but were tentatively assigned a species based upon the clustering of these sequences with other sequences of closely related, well-described species in the phylogenetic relatedness analyses described below. Different sequence types of the same presumptive species are represented in Table S1 only in instances where the differences correspond to primer and probe sequences used in the design of qPCR assays.

Bacterial cultures and DNA standards

B. fragilis (ATCC #25285), *B. thetaiotaomicron* (ATCC #29741), *B. uniformis* (ATCC #8942), and *B. vulgatus* (ATCC #8482) cells were grown in chopped meat carbohydrate broth (Remel #05047) at 37 °C for 48–72 h. Cell cultures of each strain were harvested by centrifugation for 5 min at 6000 × *g* followed by aspiration of the supernatants. Cell pellets were washed twice by suspension in 10 ml of sterile phosphate buffered saline (PBS) followed by centrifugation and aspiration of the supernatants as described above. Washed cell pellets were re-suspended in 5 ml sterile PBS. Genomic DNA isolated from 0.5 ml of these cell suspensions were used to prepare standards for the generation of respective qPCR calibration curves as previously described [11]. Briefly, the cells were lysed in a bead mill for 60 s at maximum speed and the debris were removed by centrifugation as described by Haugland et al. [20]. Approximately two/thirds (~400 μl) of the supernatants containing extracted DNA was collected in a sterile 1.7 ml low retention microcentrifuge tube and incubated for 1 h at 37 °C with 16.6 ng/μl RNase A (Sigma #D-5006). The RNase A treated DNA extracts were then purified using a commercially available silica column adsorption kit as directed by the vendor (DNA-EZ, GeneRite Inc., North Brunswick, NJ). Total DNA concentrations were determined with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies; Wilmington, DE) and rDNA target sequence copy concentrations were determined from reported estimates of the respective genome sizes and ribosomal RNA gene copy numbers per genome of these species [23].

DNA standards for the qPCR assays targeting *B. dorei*, *B. stercoris* and *Prevotella copri* and internal amplification controls (IAC) for all assays were synthesized *in vitro* by overlap extension PCR with a single site for hybridization of the unique TET-labeled TaqMan® probe sequence, UC1P1, flanked by PCR primer binding sites and cloned in a plasmid vector as previously described [40]. Standards for all qPCR analyses consisted of dilutions of the genomic or plasmid DNA preparations containing from 10 to 4×10^4 target