Forensic Science International: Genetics 7 (2013) 176–179

Contents lists available at SciVerse ScienceDirect

Forensic Science International: Genetics

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

Identification of feces by detection of *Bacteroides* genes

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ARTICLE INFO

Article history:
Received 28 April 2012
Received in revised form 18 August 2012
Accepted 24 September 2012

Keywords:
Feces
Identification
Bacteroides uniformis
Bacteroides vulgatus
Bacteroides thetaiotaomicron
Real-time polymerase chain reaction

ABSTRACT

In forensic science, the identification of feces is very important in a variety of crime investigations. However, no sensitive and simple fecal identification method using molecular biological techniques has been reported. Here, we focused on the fecal bacteria, *Bacteroides uniformis*, *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron*, and developed a novel fecal identification method by detection of the gene sequences specific to these bacteria in various body (feces, blood, saliva, semen, urine, vaginal fluids and skin surfaces) and forensic (anal adhesions) specimens. Bacterial gene detection was performed by real-time PCR using a minor groove binding probe to amplify the RNA polymerase β-subunit gene of *B. uniformis* and *B. vulgatus*, and the α-1-6 mannanase gene of *B. thetaiotaomicron*. At least one of these bacteria was detected in the feces of 20 donors; the proportions of *B. uniformis*, *B. vulgatus* and *B. thetaiotaomicron* were 95, 85 and 60%, respectively. *Bacteroides vulgatus* was also detected in one of six vaginal fluid samples, but *B. thetaiotaomicron* and *B. uniformis* were not detected in body samples other than feces. Further, we applied this method to forensic specimens from 18 donors. Eighteen anal adhesions also contained at least one of three bacteria; *B. uniformis*, *B. vulgatus* and *B. thetaiotaomicron* were detected in 89, 78 and 56%, respectively, of the specimens. Thus, these bacteria were present at a high frequency in the fecal and forensic specimens, while either *B. uniformis* or *B. vulgatus* was detected in all samples. Therefore, *B. uniformis* and *B. vulgatus* represent more appropriate target species than *B. thetaiotaomicron* for the identification of fecal material. If *B. vulgatus* and/or *B. uniformis* are detected, it is likely that the sample contains feces. Taken together, our results suggest that the use of molecular biological techniques will aid the detection of feces in forensic practice, although it is possible that the samples contained both feces and vaginal fluid.

1. Introduction

The identification of body fluid species is very important in forensic science, as facts relating to the crime can in this way be proved objectively. Moreover, identification is essential for a screening test prior to DNA genotyping. Further, the identification of feces is important evidence in particular crimes, including illegal fly tipping, harassment and sexual assault (particularly in cases of anal sexual assault); a trace of feces derived from the victim on the surface of a condom left at the crime scene can be crucial evidence. Honda and Shinohara [1] previously reported a method of feces detection by the analysis of steroids using gas chromatography. However, application of this method in forensic practice is difficult since it requires a significant quantity of sample and is complex to perform. Therefore, it is necessary to develop a sensitive and simple identification method for feces.

We reported previously a novel method of saliva identification by detecting bacteria in the oral cavity using a molecular biological technique [2,3]. On the other hand, Fleming and Harbison [4] identified vaginal fluid by the detection of *Lactobacillus* species. Further, Giampaoli et al. [5] reported the identification of vaginal fluid from various body fluids via the detection microbial signatures using a multiplex real-time PCR assay. Such PCR-based methods are highly sensitive, simple and useful in forensic practice. Similar to the oral cavity and vaginal fluid, a large number of bacterial species are present in feces. The microbial flora of the human intestinal tract comprises over 10^{11} bacterial cells per gram of colonic content, and it contains more than 400 bacterial species [6]. Moreover, a large proportion of the fecal mass consists of bacteria (~60% of fecal solids) [7]. *Bacteroides* species

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http://dx.doi.org/10.1016/j.fsigen.2012.09.006

account for as much as 30% of fecal bacteria [8]. As these bacteria are the most dominant in feces, they have been used in water pollution investigations and many studies where fecal matter was the target [9–16]. Many recent reports of the detection of bacteria from feces have been performed using real-time PCR [12–16], which allows simple and rapid detection.

Here, we focused on three *Bacteroides* species, *B. uniformis*, *B. vulgatus* and *B. thetaiotaomicron*, because they are more dominant than other *Bacteroides* species in feces [17], and reports of their existence in other body fluids are few [6,18,19]. Next, we developed a novel feces detection method based on an endpoint assay automatically judged by real-time PCR using a gene sequence specific to these bacteria. In this study, we investigated the specificity for feces of these bacteria using various body specimens (feces, blood, saliva, semen, urine, vaginal fluids and skin surfaces), and evaluated this method of feces detection using forensic samples (anal adhesions).

2. Materials and methods

2.1. Samples

All donors were healthy Japanese adults with no known diseases, including carcinoma of the gastrointestinal tract. Also, they had no history of antibiotic use, which may disturb the gut microbial flora, within 2 weeks before sample collection. Feces, blood, saliva, semen, and urine samples were collected from 20 donors and adhered to cotton swabs. Skin bacteria were collected from 20 donors by wiping the skin with a wet cotton swab; vaginal fluid samples were collected from six donors using a cotton swab. Forensic samples were collected from 18 donors by wiping the anus with a cotton swab. Informed consent was obtained from all participants who provided samples.

The bacterial strains used in this study were *B. uniformis* ATCC 8492, *B. vulgatus* ATCC 8482, *B. thetaiotaomicron* ATCC 29741, *B. fragilis* ATCC 25285, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 8100, *Staphylococcus aureus* ATCC 25923 and *Streptococcus salivarius* ATCC 13419. These strains were purchased from Microbiologics (St. Cloud, MN, USA). All *Bacteroides* species were cultured on Bacteroides Agar "Nissui" (Nissui Pharmaceutical Co., Tokyo, Japan). The streptococci were cultured on Mitis-Salivarius Agar (BHI, Difco Laboratories, Detroit, MI, USA), and the remaining bacteria were cultured on Nutrient Agar (BHI).

2.2. DNA extraction

The samples were removed from a 3 × 3-mm piece of cotton swab. DNA extraction and purification were performed using an EZ1 Investigator Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol, and elution was carried out using 50 μL of water. In the cultured bacterial samples, DNA was extracted and purified using a QIAamp® Mini Kit (Qiagen) according to the manufacturer's protocol, and elution was performed using 150 μL of water. The quantity and purity of the DNA was evaluated by means of OD_{260} and $OD_{260/280}$ measurements using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific Inc., Waltham, MA, USA). The reproducibility of the purification process was confirmed by evaluating several cultured bacterial samples using commercially available kits (EZ1 Investigator Kit, QIAamp® Mini Kit and Prepfiler DNA Extraction Kit; Life Technologies, Carlsbad, CA, USA). Further, feces generally contain polymerase inhibitors that obstruct PCR and increase the Ct-value in real-time PCR. The fecal DNA extract had no effect on the Ct-value of the TaqMan® Exogenous Internal Positive Control (IPC; Life Technologies), indicating that such inhibitors were removed by the DNA extraction and purification process.

2.3. Real-time PCR

Bacteroides uniformis, *B. vulgatus* and *B. thetaiotaomicron* were chosen as the target bacteria. Detection of bacterial genes was performed by real-time PCR using a minor groove binding (MGB) probe. Primers were designed using Primer Express® software (Life Technologies) to amplify the RNA polymerase β-subunit (*rpoB*) gene from *B. uniformis* and *B. vulgatus*, and the α-1-6 mannanase gene of *B. thetaiotaomicron* (GenBank accession numbers AY338188, AY338189 and NC004663, respectively). The selected primer target sites were compared to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST), and were checked to be complementary with the target species but not with other species. The primer sequences are shown in Table 1.

PCR was performed in 50-μL reaction mixtures containing 2× TaqMan® Universal PCR Master Mix (Life Technologies), 900 nM oligonucleotide primers, 250 nM probes, and 1 μL of template DNA. PCR amplification was performed using the 7500 Real-Time PCR System (Life Technologies) programmed for 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

A positive reaction was statistically and automatically judged using the "Presence/Absence mode" (endpoint assay) installed in the 7500 Real-Time PCR System and the TaqMan® Exogenous Internal Positive Control Reagents Kit (Life Technologies) according to the manufacturer's protocol. This system enables the identification of samples that are positive and negative for a specific target sequence, and it can discriminate two types of negative reactions: no target sequence and the presence of a PCR inhibitor. The positive threshold values were decided based on a statistical analysis of data from no template DNA and negative IPC controls.

We analyzed the DNA extract from all cultured bacterial samples by triplicate endpoint assays. The detection limits of this method were determined from the positive results of three independent experiments.

Table 1
Primer sequences used in this study.

Target	Primer	Sequence (5′–3′)	Gene
B. uniformis	Forward	GACCTGATTAACGCCAAGACAAT	*rpoB*
	Reverse	TGACAAAGCATTCGTTCCAAAG	
	Prove	FAM-TCTTCCGTTATCAATTCA-MGB	
B. vulgatus	Forward	CGATTGGTCTGGCACGTATG	*rpoB*
	Reverse	ACTTCATTGTCACGCACATTCAT	
	Prove	FAM-TCGTACCATCCGTGAGC-MGB	
B. thetaiotaomicron	Forward	TACAATTGCCACAGTACGGAACA	*a-1-6 mannanase*
	Reverse	GCTGACGAACGATGACCATAGTTA	
	Prove	FAM-ATGAGATTTCTGCCATAGCA-MGB	