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Effects of DNA Extraction Procedures on *Bacteroides* Profiles in Fecal Samples from Various Animals Determined by Terminal Restriction Fragment Length Polymorphism Analysis

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ABSTRACT

A major assumption in microbial source tracking is that some fecal bacteria are specific to a host animal, and thus provide unique microbial fingerprints that can be used to differentiate hosts. However, the DNA information obtained from a particular sample may be biased depending on the performance of the extraction procedure. In this study, we compared profiles generated by T-RFLP analysis to determine the diversity of *Bacteroides* communities in different animal hosts obtained by different DNA extraction procedures. A total of 30 feces from nine animals and three sludge samples from two wastewater treatment facilities were collected and tested to identify unique T-RFs. DNA was extracted using five different commercial DNA purification kits, amplified with FAM-labeled general *Bacteroidales* marker (Bac32F) and digested with *Hae*III for 16 hrs at 37°C. Fecal DNA was generally extracted more efficiently by the kits employing the bead-beating method; however, T-RFs profiles displayed more background noise. Profiles of T-RFs indicated that the diversity of fecal *Bacteroides* varied significantly in fecal material from the same animal source when extracted using different procedures. Therefore, the extraction procedure needs to be taken into consideration when studying the structure and composition of the microbial community as output from the different procedures may influence the perceived diversity of the sample. *Bacteroides* T-RFs were more abundant in fecal DNA from ruminants which were found to be distinctly different from the patterns derived from other animal fecal communities. Host specific T-RFs were identified in the fecal DNA from pig, deer, and sheep, regardless of the kit used for DNA extraction. The variability of T-RFs among feces from various animals could be used for identification of host-specific fingerprints in microbial source tracking studies.

EXPERIMENTAL DESIGN

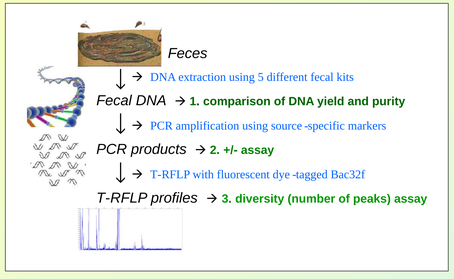

Figure 1. Experimental scheme.

Fecal samples: A total of 30 fecal samples were obtained freshly from 9 different animals including cow, chicken, pig, horse, sheep, dog, goose, goat and deer, and 3 sludge samples were collected from two wastewater plants. Each fecal sample was collected in a sterile bag using a sterilized utensil, transported on ice to the laboratory and stored at -20°C until processed. It was assumed that the three sludge samples from the wastewater treatment plant were mainly composed of human feces. Sterilize PBS buffer (10 ml, pH 7.2) was added to each fecal sample, thawed (~ 10 grams) in 50 ml tubes and homogenized using a vortex for 5 min.

DNA Extraction and PCR amplification. DNA was extracted using 5 commercial kits including Bioneer's AccuPrep™ Stool DNA Extraction Kit, Epicentre®'s ExtractMaster™ Fecal DNA Extraction Kit, Qbiogene's FastDNA® SPIN Kit for Soil, MoBio's UltraClean™ Fecal DNA Isolation Kit, and Qiagen®'s QIAamp® DNA Stool Mini Kit according to each manufacturer's instruction. Amplification was performed using GoTaq™ master mix (Promega) with primer sets in Table 1.

Table 1. Primer sets used for this study.

Primer	Sequence (5' to 3')	Expected size of PCR products (bp)	Annealing Temp. (°C)[1]	Host specificity
Bac32F	AACGCTAGCTACAGGCTT	676	58	General
Bac708R	CAATCGGAGTTCTTCGTG			
CF128F	CCAACYTTCCCGWTACTC	580[a]	53[b]	Ruminant
HF183F	ATCATGAGTTCACATGTCCG	525[a]	59[b]	Human
Bac2	GCTTGTTGCGTTCCTTGAGATAAT ACAAGCCAGGTGATACAGAAAG	274	55	Cattle
CP1-1	GGCAGGCATCAAGTCAACA TGGCAAAAGCAACTGTCATGGCA	281	61	Bird

[1] Each annealing temperature was adjusted after gradient PCR.
[a, b] calculated in a combination with Bac708R.

Terminal Restriction Fragment Length Polymorphism analysis. The PCR products generated from the amplification with carboxyfluorescein (FAM)-labeled Bac32F (IDT, Inc.) and Bac708R were used for restriction digests with *Hae*III (New England BioLabs). The reaction was carried out in a 10 µl volume containing 20-40 ng of PCR product and 10 U of the restriction enzyme at 37°C for 16 hrs.

The digested DNA was precipitated with 1 ml of 1.5 M NaOAc + 250 mM EDTA (pH 8) and 40 ml of 95% ET-OH, and the precipitate was washed twice with 70% ethanol and resuspended with Hi-Di™ Formamide and DNA Marker (GENESCAN™-600 LIZ®, Applied Biosystems). Approximately 25 fmol portions (1 ml) of restriction digest products were resolved on an ABI PRISM 3730XL® DNA sequencer (Applied Biosystems, Foster City, CA). The T-RFLP assay was performed on duplicate samples from each feces.

RESULTS

DNA Yield, Purity, and PCR Sensitivity

Table 2. Comparing DNA yield and purity generated by using 5 commercial kits: A, AccuPrep™ Stool DNA Extraction Kit; B, ExtractMaster™ Fecal DNA Extraction Kit; C, FastDNA® SPIN Kit for Soil; D, UltraClean™ Fecal DNA Isolation Kit; E, QIAamp® DNA Stool Mini Kit.

Origin of fecal material	Number of samples	DNA yield (µg/g of sample)[a]					DNA purity ($A_{260/280}$)				
		A	B	C	D	E	A	B	C	D	E
Cow	5	12.05±0.76	-	20.85±0.93	8.78±0.48	6.32±0.19	1.27±0.04	-	1.56±0.01	0.90±0.02	1.53±0.06
Chicken	3	21.75±2.20	0.50±0.07	21.06±1.50	4.97±1.20	37.09±5.06	0.71±0.10	0.42±0.08	0.61±0.01	0.99±0.04	1.98±0.04
Deer	3	3.68±0.59	1.30±0.21	62.93±4.38	18.23±5.80	13.65±2.27	1.11±0.17	1.00±0.18	1.71±0.01	1.06±0.04	1.72±0.12
Dog	3	13.16±1.90	1.86±0.22	35.29±5.20	12.65±0.87	103.94±5.18	1.47±0.11	1.54±0.29	1.74±0.02	1.39±0.03	1.95±0.02
Goat	4	29.62±3.68	1.71±0.25	170.60±19.73	9.09±1.31	30.80±1.49	1.38±0.04	1.20±0.24	1.72±0.01	0.84±0.09	1.65±0.07
Goose	3	6.07±1.30	0.48±0.08	7.74±1.10	20.95±1.33	4.87±1.00	0.69±0.14	1.01±0.44	1.17±0.07	0.52±0.05	1.28±0.22
Horse	3	2.22±0.44	-	33.20±2.85	3.84±0.40	6.74±0.63	1.51±0.40	-	1.74±0.03	0.44±0.04	1.59±0.13
Pig	3	11.21±0.87	-	36.57±2.25	7.87±1.58	20.05±1.07	1.52±0.08	-	1.74±0.03	1.22±0.08	1.75±0.04
Sheep	3	10.72±2.19	0.48±0	110.55±10.07	12.54±1.67	19.42±1.48	1.37±0.17	0.60±0	1.83±0.05	0.91±0.04	1.73±0.06
Sludge	3	4.78±0.18	5.59±0.86	31.93±3.45	5.82±0.79	15.34±4.31	1.89±0.08	1.81±0.10	1.67±0.02	1.24±0.09	1.66±0.08

[a] Values are means ± standard errors (SE).
(-) values were excluded in the analysis.

Table 3. Comparing the sensitivity of PCR assays with DNA from 5 commercial kits: A, AccuPrep™ Stool DNA Extraction Kit; B, ExtractMaster™ Fecal DNA Extraction Kit; C, FastDNA® SPIN Kit for Soil; D, UltraClean™ Fecal DNA Isolation Kit; E, QIAamp® DNA Stool Mini Kit.

1) Ruminant *Bacteroides* marker (CF128)

Origin of fecal material	A	B	C	D	E
Cow	+	+	+	+	+
Chicken	-	-	-	-	-
Deer	+	+	+	+	+
Dog	-	-	-	-	-
Goat	+	+	+	+	+
Goose	-	-	-	-	-
Horse	-	-	-	-	-
Pig	-	-	-	-	-
Sheep	+	+	+	+	+
Sludge	-	-	-	-	-

2) Human *Bacteroides* marker (Hf183)

Origin of fecal material	A	B	C	D	E
Cow	-	-	-	-	-
Chicken	+	-	+	+	+
Deer	+	-	+	-	+
Dog	+	-	+	+	+
Goat	-	-	-	-	-
Goose	-	-	-	-	-
Horse	-	-	-	-	-
Pig	-	-	-	-	-
Sheep	-	-	-	-	-
Sludge	+	+	+	+	+

3) Cattle-specific metagenomic marker (Bac2)

Origin of fecal material	A	B	C	D	E
Cow	+	-	+	+	+
Chicken	-	-	-	-	-
Deer	-	-	-	-	-
Dog	-	-	-	-	-
Goat	-	-	-	-	-
Goose	-	-	-	-	-
Horse	-	-	-	-	-
Pig	-	-	-	-	-
Sheep	-	-	-	-	-
Sludge	-	-	-	-	-

4) Bird-specific metagenomic marker (CP1-1)

Origin of fecal material	A	B	C	D	E
Cow	-	-	-	-	-
Chicken	+	-	+	+	+
Deer	-	-	-	-	-
Dog	-	-	-	-	-
Goat	-	-	-	-	-
Goose	-	-	+	-	-
Horse	-	-	-	-	-
Pig	-	-	-	-	-
Sheep	-	-	-	-	-
Sludge	-	-	-	-	-

Diversity of T-RFLP Patterns and Principal Components Analysis of T-RFLP Patterns

- Variability found in T-RFLP patterns.
- Some of ruminants T-RFs, horse T-RFs, and dog T-RFs were distinctly grouped.

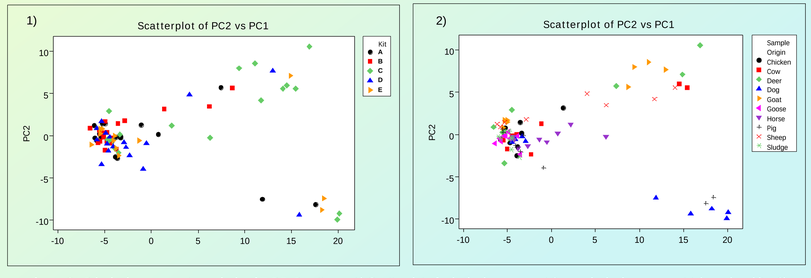

Figure 2. Principal components analysis of T-RFLP patterns: 1) Scatterplot of principal component 2 vs. principal component 1 based on kits, 2) Scatterplot of principal component 2 vs. principal component 1 based on different sample origins.

Table 4. One-way ANOVA analysis (adjusted r^2=13%; P=0.004) of T-RFLP patterns derived from five different DNA extraction kits. A, AccuPrep™ Stool DNA Extraction Kit; B, ExtractMaster™ Fecal DNA Extraction Kit; C, FastDNA® SPIN Kit for Soil; D, UltraClean™ Fecal DNA Isolation Kit; E, QIAamp® DNA Stool Mini Kit.

	Number of T-RFLP datasets	Mean ± S.D. (Peaks)
A	18	47.28 ± 33.65
B	20	38.00 ± 17.39
C	19	79.05 ± 41.46
D	18	50.78 ± 27.21
E	12	60.67 ± 44.34

Source-Specific T-RFs

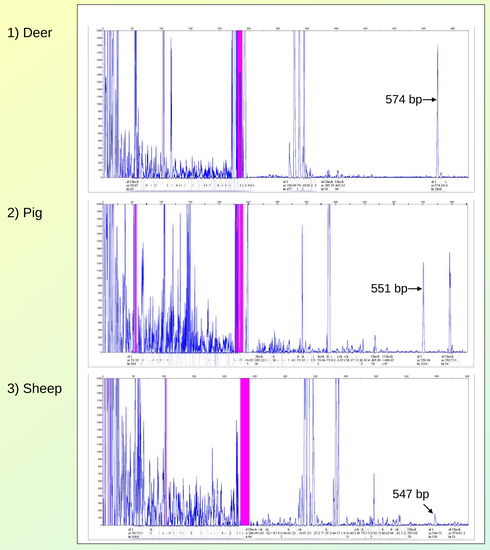

Figure 3. Peaks generated by T-RFLP analyses showing possible source-specific fragments: 1) deer, 2) pig, and 3) sheep.

CONCLUSION

1. Fecal DNA was generally extracted more efficiently by the kits employing the bead-beating method; however, T-RFs profiles from this method displayed more background noise.
2. Profiles of T-RFs indicated that the diversity of fecal *Bacteroides* varied significantly in fecal material from the same animal source when extracted using different procedures. Therefore, the extraction procedure needs to be taken into consideration when studying the structure and composition of the microbial community as output from the different procedures may influence the perceived diversity of the sample.
3. *Bacteroides* T-RFs were more abundant in fecal DNA from ruminants, some of which were found to be distinctly different from the patterns derived from other animal fecal communities.
4. Host specific T-RFs were identified in the fecal DNA from deer, pig, and sheep, regardless of the kit used for DNA extraction.

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