Biotechnology and Bioprocess Engineering 22: 30-36 (2017)
DOI 10.1007/s12257-016-0630-6

RESEARCH PAPER

Potential Application of Genetically Identical Somatic Cell Nuclear Transfer-cloned Dogs for Gastrointestinal Microbiota Analysis

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Received: 2 December 2016 / Revised: 21 December 2016 / Accepted: 28 December 2016

Abstract This study investigated the gastrointestinal microbiota in three genetically identical cloned dogs (A, B and C) by somatic cell nuclear transfer. We collected feces from three cloned dogs and their feed to investigate gastrointestinal microbiota using both culture-dependent and culture-independent methods. A total of 962 strains from the feces of cloned dogs were isolated using aerobic and anaerobic culture methods. The dominant microorganisms were *Enterococcus faecalis* and *Enterococcus faecium* in all fecal samples. In particular, the fecal sample from cloned dog C had similar proportions of three species (*E. faecalis*, *E. faecium* and *Lactobacillus murinus*). In all, 29 DNA fragments were identified by PCR-denaturing gradient gel electrophoresis (DGGE) analysis. The highest DGGE band intensities were for *E. faecalis* from cloned dogs A and C and for *Clostridium sordellii* from cloned dog B, with relative intensities of 15.2, 17.7 and 14.4%, respectively. The other strains identified from the cloned dogs were *Chryseobacterium soldanellicola*, *Escherichia coli*, *L. murinus*, *Streptococcus alactolyticus*, *Weissella confusa* and uncultured bacterium. Some microbes isolated from the fecal samples, including *C. soldanellicola* and *W. confuse*, were derived from the feed. Overall, gastrointestinal microbiota of all genetically identical cloned dogs, maintained under the same environmental and feeding conditions, showed similar profiles in terms of species diversity analyzed by PCR-DGGE, although there were proportional differences in the amounts of bacterial species. To our knowledge, this is the first report to investigate and compare gastrointestinal microbiota of three genetically identical dogs.

Keywords: gastrointestinal microbiota, genetically identical dogs, denaturing gradient gel electrophoresis, 16S rRNA sequencing

1. Introduction

Somatic cell nuclear transfer (SCNT) can produce genetically identical animals. SCNT can be applied to preserve endangered species and produce transgenic models [1-3]. Elimination of genetic influences using genetically identical SCNT clones can decrease inter-individual variability, thereby enabling investigation of gastrointestinal microbiota. Previously SCNT-cloned pigs were used in studies examining metabolomics, phenotypic characteristics and obesity and relating these properties to gastrointestinal microbes [4-6]. Dogs are an optimal research model species for human diseases because their anatomy and physiology are similar to those of humans. In particular, multiple genetic diseases are common to dogs and humans [7].

The gastrointestinal environment is a complex microbial ecosystem influenced by the host, ingested dietary compositions and microorganisms [8]. Gastrointestinal microbes play a vital role in health of the host by stimulating the immune system, in development of gut structures, in defense against pathogens and in providing nutritional benefits to the host [9]. Previous studies on the gastrointestinal microbiota of dogs focused on enumeration and identification of microorganisms cultivated directly from feces [10,11]. However, because fewer than 1% of all

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bacterial cells in nature can be detected through culture-based approaches [12,13], recent studies of the bacterial community utilized culture-independent approaches, with analysis of DNA or RNA, enabling identification of microorganisms that could not be cultured or were present in very low numbers [14]. One approach, denaturing gradient gel electrophoresis (DGGE) was used to assess fecal microbiota in humans, including neonates [15], monozygotic twins [16] and adults [17], and in animals, including cats [18], chickens [19], earthworms [20], horses [21], piglets [22] and rabbits [23]. Many investigators have used DGGE, making it a well-established technique for studying microbial diversity.

The aim of this study was to investigate the status of gastrointestinal microbiota among genetically identical SCNT dogs, maintained under equivalent environmental and feeding conditions. We analyzed the bacterial community in feces from three cloned dogs, as well as in feed given to the dogs, using a culture-based approach to examine live cells and PCR-DGGE analysis to investigate microbial diversity.

2. Materials and Methods

2.1. Production of somatic cell cloned dogs and diets

All animal operating procedures used in this study were approved and performed in accordance with the guidelines of the National Institute of Animal Science Animal Care and Experimentation Committee in Republic of Korea (2013-045). We previously produced four somatic cell-cloned Labrador retriever dogs [24] and three SCNT-cloned dogs from the same surrogate mother were used in our study. For the production of SCNT-cloned dogs, adult fibroblast cells were isolated by an ear skin biopsy of a 7 year old male Labrador retriever dog. *In vivo* matured oocytes were recovered from naturally estrous female dogs and nuclei were removed. A donor fibroblast cell was transferred to the perivitelline space of the recipient enucleated oocyte, as described previously [25]. The couplet of the donor cell and cytoplast was fused by two electrical pulses of direct current (72 V for 15 ms each). Fused embryos were subsequently activated with 1.9 mM 6-dimethylaminopurine (Sigma-Aldrich, St. Louis, MO, USA.) for 4 h. Soon after activation, 15 embryos were surgically transferred into the oviducts of spontaneous estrous surrogate mothers. At 60 days after embryo transfer, SCNT-cloned puppies were delivered by caesarean section. Somatic cell-cloned puppies were nursed by the same surrogate mother until 6 weeks after birth. Weaned puppies were fed a standard dog-diet of Royal Canin Labrador Retriever 33 Junior for 6 weeks.

Genomic DNA was obtained from cultured adult fibroblasts, the cloned dog and the surrogate mother and analysis indicated that the three cloned dogs were identical to donor cells and different from the surrogate mother. The following 14 markers, originally derived from dogs, were selected for analysis: FH2537, FH3005, FH3027, FH3381, FH3399, FH2584, FH2712, FH3058, FH2054, FH2079, FH2790, FH3921, REN197E16 and REN10O23 (Supplemental data).

2.2. Isolation of microorganisms

Samples of fresh feces (10 g) from three 2-month-old healthy and genetically identical SCNT-cloned dogs (cloned dogs A, B and C) and feed (10 g) were transferred into sterile stomacher bags with 90 mL sterile 0.85% NaCl solution and mixed for 5 min by a stomacher (Seward Laboratory Systems Inc., Bohemia, NY, USA). After 10-fold serially diluting 1 mL of each suspension, diluents were spread onto tryptic soy agar (Becton, Dickinson and Company, Sparks, MA, USA) and MRS agar (Becton, Dickinson and Company). Agar plates were incubated at 40°C for 48 h under aerobic or anaerobic conditions.

2.3. Extraction of DNA and PCR

Aliquots (1 mL) of homogenized feces and feed samples were centrifuged at 8,000 × *g* for 15 min and the pellets washed with sterile phosphate buffered saline (pH 7.0) and resuspended in 1 mL TE buffer (10 mM Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). DNA was extracted from pellets using a DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA fragments were amplified using the universal PCR primers 341F forward (5′-CCTACGGGA GGCAGCAG-3′) and 518R reverse (5′-ATTACCGCG GCTGCTGG-3′) [28]. For DGGE analysis of the PCR products, a 40-nucleotide GC-rich sequence (GC-clamp, 5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGG-3′) was appended to the 5′ end of the primer [27]. PCR amplification was performed with using a Mastercycler gradient (Eppendorf, Hamburg, Germany). The PCR reaction mixture (20 μL) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.5 U *Taq* polymerase, 0.4 mM dNTPs, 20 pmol of each primer, 1 L template DNA (20 μg/mL) and sterile water. An initial denaturation at 95°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 45 sec, annealing at 52°C for 45 sec and extension at 72°C for 1 min) and a final extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gels with 1 × TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.0). Gels were stained with ethidium bromide (0.5 μg/mL) and bands visualized with an ultraviolet transilluminator

(Korea Bio-Tech Co., Seoul, Korea).

2.4. DGGE analysis

DGGE analysis was performed using a DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). DGGE conditions were between 30 and 65% denaturing gradient (100% denaturing solution corresponds to 7.0 M urea (Sigma Chemical Co., USA) and 40% (v/v) formamide (Sigma Chemical Co., USA) and an 8% (w/v) polyacrylamide gels (Acryl/Bis 29:1, 40% (w/v) solution; Amresco, Solon, OH, USA). Electrophoresis was performed in 0.5 × TAE buffer initiated at a voltage of 20 V for 30 min, and then at a voltage of 60 V for 12 h at 60°C. After electrophoresis, gels were stained in 1 × TAE buffer containing a 1:10,000 dilution of GreenStar nucleic acid staining dye (Bioneer Co., Seoul, Korea) for 20 min and bands visualized with a UV transilluminator (Korea Bio-Tech Co.). The intensities of bands in the DGGE gel were analyzed by Gel-Pro Analyzer software (Media Cybernetics Inc., Bethesda, MD, USA).

2.5. Determination of nucleotide sequences

16S rDNA gene sequences from isolates and DGGE gels were sequenced using a PCR-based technique. The 16S rDNA fragments from isolates were partially amplified with universal primers (27F: 5′-AGAGTTTGATCATGG CTCAG-3′; 1492R: 5′-GGATACCTTGTTACGACTT-3′). Polyacrylamide blocks containing the DGGE bands were excised with a surgical blade (Feather Safety Razor Co., Ltd., Osaka, Japan) and placed into sterilized Eppendorf tubes. DNA from each band was extracted using a QIAEX II gel extraction kit (Qiagen), according to the manufacturer's

Table 1. Identification of microorganisms isolated from the feces and feed of three genetically identical cloned dogs

Samples	Medium	Closest relative	GenBank accession no.	Identity %	Proportion (colonies)
cloned dog A	TSA	*Enterococcus faecalis*	JN102567	98	88.2% (67/76)
		Escherichia coli	NR102804	98	11.8% (9/76)
	MRS	*Enterococcus faecalis*	JN102567	97	83.0% (68/82)
		Enterococcus faecium	KF149320	97	17.0% (14/82)
cloned dog B	TSA	*Enterococcus faecium*	KF149320	98	94.2% (195/207)
		Escherichia coli	NR102804	97	5.3% (11/207)
		Chryseobacterium soldanellicola	EU834270	95	0.5% (1/207)
	MRS	*Enterococcus faecium*	KF149320	98	94.0% (228/242)
		Enterococcus faecalis	JN102567	97	6.0% (14/242)
cloned dog C	TSA	*Lactobacillus murinus*	EF445113	98	49.0% (103/212)
		Enterococcus faecalis	JN102567	97	46.0% (97/212)
		Escherichia coli	NR102804	97	5.7% (12/212)
	MRS	*Enterococcus faecalis*	JN102567	98	51.0% (102/200)
		Enterococcus faecium	KF149320	97	49.0% (98/200)
Feed	TSA	*Brevibacterium stationis*	FJ573175	97	50.0% (7/14)
		Chryseobacterium soldanellicola	EU834270	97	28.6% (4/14)
		Weissella confusa	HQ711354	97	21.4% (3/14)

Isolates were selected on tryptic soy agar and MRS agar plates, with 14–242 colonies each, and were identified by 16S rDNA gene sequence analysis. The highest similarity matches are presented. Basic Local Alignment Search Tool (BLAST) homology represents the percent identity and accession number of the sequence of the closest relative in the GenBank database.

instructions. Extracted DNA was re-amplified by PCR using 20 pmol of 341F (without the GC-clamp) and 518R primers. The re-amplified PCR products to be used for sequencing were purified with a QIAquick PCR purification kit (Qiagen). Purified PCR products were ligated into a T vector (Invitrogen, Carlsbad, CA, USA) and DNA sequencing was performed with an ABI 377 genetic analyzer (Applied Biosystems, Foster, CA, USA).

3. Results and Discussion

Quantitative and qualitative data describing canine gastrointestinal microbiota, obtained using cultured approaches, are well documented. However, there is a scarcity of data obtained using molecular analyses, especially of the gastrointestinal microbiota of cloned dogs using SCNT. Previously, we produced three genetically identical puppies from a surrogate mother [26]. Microsatellite analysis using 1–14 markers confirmed that the puppies were identical to the donor cell line used (Supplement data). To establish a better understanding of the gastrointestinal microbiota of genetically identical cloned dogs, dog feces were analyzed using culture-dependent and culture-independent (DGGE analysis) approaches.

3.1. Culture-dependent analysis of microbiota

Microbes from the fecal samples of three cloned dogs produced 2.1 ~ 7.6 × 10^8 colony forming units/g under aerobic conditions and 2.0 ~ 8.2 × 10^8 colony forming units/g under anaerobic conditions (data not shown). The plate count obtained from the feed sample was 1.4 × 10^3 colony forming units/g under aerobic conditions (data not shown). A total of 76 ~ 212 and 14 pure cultures were isolated from feces and the feed sample, respectively, under aerobic conditions. There were 82 ~ 242 pure cultures isolated from the fecal samples under anaerobic conditions.

Isolates identified are shown in Table 1. The predominant species isolated from feces of the three cloned dogs were *Enterococcus faecalis* and *Enterococcus faecium*. *E. faecalis* is a commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. Occasionally, *E. faecalis* can cause life-threatening infections in humans, particularly in the nosocomial environment related to antibiotic resistance [28]. *E. faecium* can be commensal in the human or animal intestine, but it may also be pathogenic, causing diseases such as neonatal meningitis [29]. In the fecal sample from cloned dog C, *L. murinus* was one of the predominant microorganisms (49%) along with *E. faecalis* and *E. faecium*, whereas it was not isolated alive in the other fecal samples. *L. murinus* is present in human and dog gastrointestinal tracts, where it represents a small proportion of the gut flora [30,31]. Because probiotics, including lactic acid bacteria such as *Lactobacillus* species, have health benefits in humans and animals and help maintain optimal health by balancing probiotic and harmful bacteria [9], cloned dog C might have had healthier intestines than the other dogs.

3.2. DGGE analysis of microbiota

The culture-dependent technique frequently fails to accurately classify various microorganisms inhabiting host gastrointestinal tracts because of low selectivity and long culture periods. Therefore, in parallel with the culture method, we used DGGE analysis of PCR-amplified 16S rRNA fragments, directly extracted from the feces of cloned dogs and the feed, to investigate bacterial community structures.

Band patterns in DGGE gels showed profiles indicating similar bacterial community structures in the three cloned

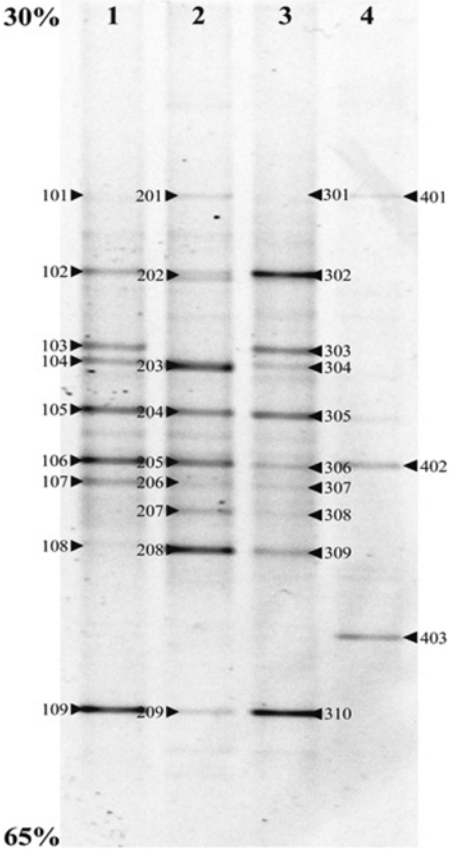

Fig. 1. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA fragments from the following samples: 1, feces from cloned dog A; 2, feces from cloned dog B; 3, feces from cloned dog C; 4, feed. PCR amplification of 16S rRNA gene sequences was performed with the primers $341F^{GC}$ and 518R (see text for sequences). Denaturing gradient gel electrophoresis (DGGE) was performed with a 30 ~ 65% denaturing gradient. Arrows indicate the bands that were excised and sequenced. See Table 2 for identification.

dogs, although band intensities differed (Fig. 1). The 16S rDNA sequences and their similarity with reference sequences enabled identification of the species represented by the DGGE bands (Table 2). In the fecal sample from cloned dog A, there were three sharp bands with high intensities (105, 106 and 109), corresponding to *E. coli*, *Chryseobacterium soldanellicola* and *E. faecalis*, respectively. Among the nine DGGE bands in the fecal sample from cloned dog B, bands corresponding to *E. faecium*, *C. soldanellicola* and *Clostridium sordellii* had high intensities (above 9%). In the fecal sample from cloned dog C, bands corresponding to *L. murinus* and *E. faecalis* had the highest intensities. All fecal samples from the cloned dogs included uncultured bacterium, banding in the same positions in DGGE gels (107, 206 and 307).

The clone library contained nine, nine and ten and three different 16S rDNA sequences from cloned dogs A, B and C and from the feed, respectively. Among the nine species identified, not including the uncultured bacterium, seven species (*W. confuse*, *L. murinus*, *E. faecium*, *E. coli*, *C. soldanellicola*, *C. sordellii* and *E. faecalis*) were found in samples from all three dogs. The other two species, *Streptococcus alactolyticus* and *Lactobacillus animalis*, were found in two of the three cloned dogs. In general, more than 10 bacterial phyla including *Firmicutes*, *Bacteroidetes*, *Proteobacteria, Actinobacteria*, and *Fusobacteria* constitute approximately 99% of all gut microbiota in regular (non-cloned) dogs [32,33]. The species detected in our study were similar to those described in previous studies, in which microorganisms, including *Bacteriodes*, *Clostridium*,

Table 2. Diversity of microorganisms identified from the feces and feed of three genetically identical coned dogs, analyzed by denaturing gradient gel electrophoresis

Lane*	Band number*	16S rRNA sequence results	GenBank accession no.	BLAST homology, %	Band intensity,%
Lane 1 (cloned dog A)	101	*Weissella confusa*	HQ711354	97	1.36
	102	*Lactobacillus murinus*	EF445113	97	3.49
	103	*Streptococcus alactolyticus*	NR041781	97	4.54
	104	*Enterococcus faecium*	KF149320	98	3.84
	105	*Escherichia coli*	NR102804	98	9.05
	106	*Chryseobacterium soldanellicola*	EU834270	95	12.2
	107	*Uncultured bacterium*	GQ233027	97	4.59
	108	*Clostridium sordellii*	KC818617	97	1.40
	109	*Enterococcus faecalis*	JN102567	98	15.2
Lane 2 (cloned dog B)	201	*Weissella confusa*	HQ711354	97	2.43
	202	*Lactobacillus murinus*	EF445113	98	3.19
	203	*Enterococcus faecium*	KF149320	97	13.4
	204	*Escherichia coli*	NR102804	98	6.08
	205	*Chryseobacterium soldanellicola*	EU834270	96	9.47
	206	*Uncultured bacterium*	GQ233027	97	2.40
	207	*Lactobacillus animalis*	HQ293063	97	4.15
	208	*Clostridium sordellii*	KC818617	97	14.4
	209	*Enterococcus faecalis*	JN102567	98	1.57
Lane 3 (cloned dog C)	301	*Weissella confusa*	HQ711354	97	1.42
	302	*Lactobacillus murinus*	EF445113	99	15.7
	303	*Streptococcus alactolyticus*	NR041781	98	4.99
	304	*Enterococcus faecium*	KF149320	97	2.08
	305	*Escherichia coli*	NR102804	98	6.18
	306	*Chryseobacterium soldanellicola*	EU834270	97	2.77
	307	*Uncultured bacterium*	GQ233027	97	2.12
	308	*Lactobacillus animalis*	HQ293063	96	1.52
	309	*Uncultured Clostridium sp.*	DQ856765	96	3.96
	310	*Enterococcus faecalis*	JN102567	99	17.7
Lane 4 (Feed)	401	*Weissella confusa*	HQ711354	96	2.02
	402	*Chryseobacterium soldanellicola*	EU834270	96	6.18
	403	*Brevibacterium stationis*	FJ573175	97	7.23

*Each number corresponds to the bands indicated in Fig. 1.
The 16S rRNA fragments from the DGGE bands were aligned with GenBank reference sequences (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Lactobacillus and *Enterobacteriaceae*, were the predominant bacterial groups in the gastrointestinal tracts of regular dogs [34,35]. However, regular dogs showed a very unique and individual microbial profile and diverse microbial species, including *Streptococci, Bacteroides*, *Bifidobacterium*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Peptococcus* and *Lactobacillus*, in their gastrointestinal tracts [36,37]. The number of bands in DGGE profiles was 11–34 in fecal samples of 17 beagle dogs [18], whereas it was 9–10 in that of three cloned dogs. Of the three species (*W. confuse*, *C. soldanellicola* and *Brevibacterium stationis*) detected in the feed sample, *W. confuse* and *C. soldanellicola* were also detected in fecal samples from all cloned dogs (Table 2). The results suggested that the feed given to the dogs affected the microbial community of the host. It was reported that diet composition affected the intestinal microflora of dogs. This was despite individual variability and many intestinal microflora differences among dogs, even when similarly housed and fed identical diets [11,38].

Overall, in our study, profiles obtained through DGGE analysis revealed correlations among bacterial community structures in the gastrointestinal tracts of the cloned dogs. There was a high degree of similarity in the gastrointestinal microbiota of the dogs when analyzed by DGGE (Fig. 1). It was reported that host genome influenced the microbiota in the guts of human monozygotic twins [39]. The gastrointestinal microbiota of human monozygotic twins was slightly more similar than in unrelated individuals, suggesting that genetically identical individuals harbor similar gut microbiota [40]. Therefore, SCNT-cloned dogs could provide a relatively homogenous experimental animal model to minimize genetic influences and inter-individual variations.

4. Conclusion

Genetically identical SCNT-cloned dogs have very similar gastrointestinal microbial communities when analyzed using PCR-DGGE. More diverse species were detected using DGGE analysis than using conventional culture-based methods, primarily because DGGE analysis could detect bacteria that cannot be cultured. Among the nine species analyzed by DGGE, seven were detected in fecal samples from all cloned dogs. The other two species, *S. alactolyticus* and *L. animalis*, were found only in cloned dogs A and C, and cloned dogs B and C, respectively. Some microorganisms in the feed survived in the gastrointestinal tracts of dogs after digestion. To our knowledge, this is the first report suggesting that three genetically identical dogs have similar gastrointestinal microbiota. It is possible that more diverse species would be identified using advanced molecular tools such as next-generation sequencing. Therefore, further studies will be required to identify more diverse profiles and phylogenetic characterizations of the microbiota from the gastrointestinal tracts of SCNT-cloned dogs.

Acknowledgement

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009221)" Rural Development Administration, Republic of Korea.

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