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Impact of enrofloxacin on the human intestinal microbiota revealed by comparative molecular analysis

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ABSTRACT

The indigenous human intestinal microbiota could be disrupted by residues of antibiotics in foods as well as therapeutically administered antibiotics to humans. These disruptions may lead to adverse health outcomes. To observe the possible impact of residues of antibiotics at concentrations below therapeutic levels on human intestinal microbiota, we performed studies using *in vitro* cultures of fecal suspensions from three individuals with 10 different concentrations (0, 0.1, 0.5, 1, 5, 10, 15, 25, 50 and 150 μg/ml) of the fluoroquinolone, enrofloxacin. The bacterial communities of the control and enrofloxacin dosed fecal samples were analyzed by denaturing gradient gel electrophoresis (DGGE) and pyrosequencing. In addition, changes of functional gene expression were analyzed by a pyrosequencing-based random whole-community mRNA sequencing method. Although each individual had a unique microbial composition, the communities of all individuals were affected by enrofloxacin. The proportions of two phyla, namely, Bacteroidetes and Proteobacteria, were significantly reduced with increasing concentrations of enrofloxacin exposure, while the proportion of Firmicutes increased. Principal Coordinate Analysis (PCoA) using the Fast UniFrac indicated that the community structures of intestinal microbiota were shifted by enrofloxacin. Most of the mRNA transcripts and the anti-microbial drug resistance genes increased with increasing concentrations of enrofloxacin. 16S rRNA gene pyrosequencing of control and enrofloxacin treated fecal suspensions provided valuable information of affected bacterial taxa down to the species level, and the community transcriptomic analyses using mRNA revealed the functional gene expression responses of the changed bacterial communities by enrofloxacin.

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1. Introduction

The human gastrointestinal tract ecosystem consists of complex microbial communities and the indigenous microbiota play an important role in maintaining and influencing human health [1,2]. A variety of external factors, including diet and antibiotic exposure, can disturb the composition of indigenous intestinal microbiota and may contribute to susceptibility, development and exacerbation of disease [1,3–6]. The shift in the composition of the intestinal microbiota has been associated with obesity, type 1 and 2 diabetes, some allergies and inflammatory bowel diseases in humans and animal models [7–10].

The administration of antibiotics to food producing animals could result in antibiotic residues in the edible tissues and meat products if illegally used or withdrawal times are not adhered [11–16]. Maximum residue limits (MRLs) are established to reflect the maximum concentration of residue in a food that is legally permitted when the veterinary drug is used according to the approved recommended conditions [17]. Regulatory authorities control the MRL of antibiotics in food and human consumption of violative residues is a rare event, however it is still important to investigate the influence of low concentration of antibiotic on human intestinal microbiota as part of the risk assessment process. Because therapeutic doses of antibiotics have known to shift the microbial population in the gastrointestinal tract [1,5], less is known about the impacts of lower-level antibiotic concentration than therapeutic doses on the human intestinal microbiota and the changes of microbial gene expression. In addition, regulatory authorities are seeking *in vitro* and *in vivo* models and sensitive methods for examining effects of low concentrations of antibiotics on the human microbiota [17].

Enrofloxacin is a fluoroquinolone anti-microbial agent with a broad spectrum of anti-microbial activity marketed for veterinary medicine. In 2005, the FDA withdrew approval of Baytril (enrofloxacin) for use in poultry in the U.S.A., since it could select for

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fluoroquinolone–resistant *Campylobacter* [18]. However, enrofloxacin is still approved for use in some food producing animals and companion animals [19–22]. Enrofloxacin is de-ethylated to ciprofloxacin in cows and dogs, which is more active against certain bacteria than enrofloxacin [23–25]. Ciprofloxacin is commonly used in human medicine to treat infectious diseases caused by aerobic and facultative anaerobes, and a few studies have investigated the impact of ciprofloxacin and enrofloxacin on the human intestinal microbiota [1,26–28]. However, less is known about the effects of low concentrations of enrofloxacin on human intestinal microbiota.

Recent advances in molecular approaches for microbial community analysis have led to a more detailed understanding of the complexity of the human intestinal microbiota and their community structure compared to conventional microbiological culture methods. Furthermore, the metatranscriptomic analyses (RNA-seq) based on high-throughput sequencing can provide information about the gene expression response of microbial taxa to varying environmental conditions [14,29,30]. In contrast to microarray-based techniques which provide information only on known gene sequences, RNA-seq allows the characterization of both known and unknown gene transcripts. These high-throughput approaches with rRNA and mRNA genes were applied in this investigation for evaluating the influence of enrofloxacin on the intestinal microbiota.

In this study, we assessed the impact of enrofloxacin using denaturing gradient gel electrophoresis (DGGE) to rapidly monitor changes in the intestinal microbiota, 16S rRNA gene-based pyrosequencing to analyze the perturbation of the microbial community at each phylogenetic level, and an RNA-seq approach to investigate the differences in functional gene expressions of influenced intestinal microbiota.

2. Materials and methods

2.1. Culture conditions for enrofloxacin treatment of fecal suspensions

The fecal inocula were obtained from 3 individuals coded A, B and C. These volunteers were healthy males between 50 and 60 years old with no antibiotics administered within the 6 months before sampling. The storage, incubation and culturing of fecal suspensions followed a recently published method of *in vitro* culture conditions for human intestinal microbiota [31]. Briefly, feces were diluted with anaerobic Maximum Recovery Diluent (MRD; LabM IDG, Bury, UK) buffer to 25% concentration (w/v). 3% of fecal suspension (final concentration; w/v) from 25% fecal dilution were inoculated to low concentration carbohydrate medium (10 ml of final volume) supplemented with 1% fecal supernatant, and cultured anaerobically at 37 °C for 18 h. The use of human fecal samples was approved by the FDA Research Human Subjects Committee (approval number 09-033T).

Enrofloxacin (Fluka Biochemika, distributed by Sigma–Aldrich Corporation, St. Louis, MO, USA) was dissolved in KOH under anaerobic N_2 gas and the concentrated stock solution (10×) was prepared. The impact of enrofloxacin on the human intestinal microbiota was investigated in triplicate experiments with 10 different concentrations (0, 0.1, 0.5, 1, 5, 10, 15, 25, 50 and 150 μg/ml).

2.2. Nucleic acid extraction and DGGE analysis

Genomic DNA and total RNA were extracted from 1 ml of each sample (at 0 h and 18 h) using a total RNA extraction kit with a DNA elution accessory kit (MoBio Laboratories, Carlsbad, CA, USA). The V3 region of the 16S rRNA genes was amplified using primers GC-clamp-340f (5′-TCC TAC GGG AGG CAG CAG-3′) and 518r (5′- ATT ACC GCG GCT GCT GG-3′) for conducting DGGE [32,33]. The PCR reaction was performed using a Mastercycler gradient instrument (Eppendorf, Hauppauge, NY, USA), in a final volume of 50 μl with 10X Taq buffer, dNTP mixture (Takara, Shiga, Japan), 10 μM of each primer (MWG-Biotech, Ebersberg, Germany), 2 U of Taq polymerase (Ex Taq; Takara). After initial denaturation at 94 °C for 5 min, amplification was conducted by 30 cycles of denaturation (30 s, 94 °C), primer annealing (30 s, 55 °C), and extension (30 s, 72 °C), with a final extension step of 7 min at 72 °C. The PCR product was confirmed by using 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The extraction and amplification of genomic DNA from triplicate culture samples were conducted independently. The DNA concentration was determined on a NanoDrop 1000 instrument (NanoDrop Technologies, Wilmington, DE, USA). Equal amounts of amplified products from each sample were loaded on the DGGE gel. The denaturing gradient ranged from 40% to 65% and the run was at 70 V for 16 h 30 min at 60 °C, using the Dcode system (BioRad). The profiles of DGGE gels were visualized by ethidium bromide staining and photographed using the Gel Doc system (BioRad). Normalization and analysis of gel profiles were conducted using the BioNumerics program version 6.0 (Applied Maths, St.-Martens-Latem, Belgium). The DGGE profiles of triplicate samples were compared to each other to evaluate the reproducibility of experiments. To identify the sequences of changed bands, bands were excised from the gels, reamplified with GC-clamp free primers (340f and 518r), purified, cloned, and sequenced. The sequences from excised DGGE bands were submitted to the EMBL database under accession numbers from HE565611 to HE565646.

2.3. Pyrosequencing analysis of 16S rRNA genes

The change of microbial community composition was determined using pyrosequencing analysis at each phylogenetic level. 16S rRNA genes were amplified from genomic DNA of a total of 30 samples (1 control and 9 different concentrations of enrofloxacin treated samples for each individual) using barcoded primers. The effect of incubation time on microbial diversity for 18 h cultivation was recently reported [31]. The amplification, sequencing, and basic analysis were performed according to the methods described by Chun et al. [34] using a 454 GS FLX Titanium Sequencing system (Roche, Branford, CT, USA). Any reads containing two or more ambiguous nucleotides, low quality score (average score < 25), or reads shorter than 300bp, were discarded. After chimera check, the taxonomic classification of each read was assigned against the extended EzTaxon database (http://www.eztaxon-e.org) [35]. To describe sequences closely related to uncultured sequences, the representative phylotypes of uncultured sequences were contained in the extended EzTaxon database. The statistical analyses of microbial communities were performed using the Mothur program [36]. The richness and evenness of samples were determined by Chao1 estimation and Shannon diversity index at the 3% distance, which characterized the richness at the species level [37,38]. The similarity of community structure between samples was obtained using Bray–Curtis similarity and Jaccard similarity, and the significances of difference between community structures were analyzed using Libshuff analysis. To compare samples with different read numbers, the sizes of different samples were normalized by random subtraction. The principal coordinate analysis (PCoA) and Fast UniFrac analysis were conducted using the CLcommunity software (Chunlab, Inc., Seoul, Korea) following the algorithm developed by Hamady et al. [39]. The correlation analyses of