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Full Length Research Paper

Effects of levofloxacin hydrochloride on the intestinal microbiota of BALB/c mice by PCR-DGGE

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Levofloxacin hydrochloride (LH) is recommended for use in the treatment of infectious diseases, but its side effect has not been thoroughly investigated, especially on the intestinal tract. LH was tested of its side effects on effect to intestinal microbiota of BALB/c mice in this study. The similarity, diversity, and sequence analysis of the dominant bands for different drug administration periods were carried out by denaturing gradient gel electrophoresis (DGGE) profiles. The total amount of 16S rRNA gene was increased after drug administration, the bacterial composition and structure could be divided into different clusters for different drug administration periods distinctly. *Prevotella amni*, *Porphyromonas uenonis*, *Bacteroides coprophilus*, *Streptococus sanguinis*, *Bacteroides sp.*, *Clostridium butyricum str.* and *Leptotrichia hofstadii* were dominant organisms. This study revealed a significant change of bacterial composition of microflora on the tested mice for LH.

Key words: Levofloxacin hydrochloride, side effects, intestinal microflora, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Levofloxacin hydrochloride (LH), the active L-isomer of ofloxacin, is a widely used fluoroquinolone for oral administration, it has been previously acknowledged as a “new generation” of fluoroquinolones since it has a broader antibacterial spectrum and improved pharmacokinetics when compared to ciprofloxacin, the first fluoroquinolone used for systemic infections (North et al., 1998). LH has the activity against Gram-positive and Gram-negative bacteria as well as atypical infections, such as *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* (Karlowsky et al., 2003; Jones et al., 2003; Critchley et al., 2002; Jones et al., 1999; Flynn et al., 1996). It has been documented both *in vitro* and clinically (Wexler et al., 1998; Goldstein et al., 1995; Goldstein et al., 1999).

Levofloxacin has become one of the most commonly prescribed fluoroquinolone antimicrobials in China during the past 14 years, with activity against bacteria that cause respiratory, skin, and genitourinary tract infections (Noel, 2009), but the side effects accompanying the drug administration are, especially intestinal tract, such as abdominal pain, diarrhea, astriction (Sprandel and Rodvold, 2003; Gisbert et al., 2008; Li et al., 2010).

DGGE based on sequence variability in 16S rRNA genes has been used. This molecular technique has been successfully applied to identifying sequence variations in a number of genes from several different organisms (Muyzer et al., 1993), DNA fragments of the same length but with different base-pair sequence can be separated (Fischer and Lerman, 1979). The objective of this study was to investigate the effects of LH on the intestinal microflora of BALB/c mice by PCR-DGGE.

The similarity, diversity, and sequence analysis of the dominant bands for different drug administration periods were carried out. The investigation of intestinal microflora affected by LH would explore the risk factors associated with side effect of intestinal tract.

MATERIALS AND METHODS

Subjects and preparation of samples

Ten female BALB/c mice aged 6 weeks of SPF grade were supplied by Animal Lab Center of Dalian Medical University; certificate of

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quality number is SCXK (Liao) 2008-0002. Dosage (*d*) was according to the Meeh-Rubner conversion formula between human and mouse:

d_{mouse} (mg/kg) = d_{human} (mg/kg) × (K_{mouse} / K_{human})

Where, *K* was conversion factor, K_{mouse} = 1 ; K_{human} = 0.11.

So, LH was given at 65 mg/kg for 10 days, and stopping drug for 7 days. Fecal samples were collected per mice in each group at 0d (Normal), 3d (I), 10d (II) of drug administration periods, and stopping drug 7d (III) period, respectively, and preserved -80°C.

Deoxyribonucleic acid (DNA) extraction

DNA was extracted from fecal samples with E.Z.N.A® Stool DNA Kit (OMEGA, BIO-TEK, USA) in accordance with the manufacturer's instructions. The amount and quality of DNA extracts were analyzed by electrophoresis of 1% agarose gel containing ethidium bromide, and compared to a molecular weight standard (1 kb). DAN extracts were preserved at -20°C.

Polymerase chain reaction (PCR) amplification

PrimersGC-357f(space)(5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGACGGGGGGCCTACGGGAGGCAGCAG), at its 5' end an additional 40-nucleotide GC-rich sequence (GC clamp), and 518r (5'-ATTACCGCGGCTGCTGG) (Muyzer et al., 1993) were used to amplify the V3 region of bacterial 16S rRNA (Primers were synthesized by TaKaRa Biotechnology Co., Ltd.). PCR amplification was performed with FerroTec Thermal Cycler (HangZhou Dahe Thermal-magnetics Electronics Co., Ltd.) as follows: 3 µl purified genomic DNA as template, 10×^{Ex}Taq buffer (Mg^{2+} plus) 2.5 µl, dNTP mixture 4 µl, BSA (1 mg/mL) 2.5 µl, 10 pmol of each primer, 1.25 U of ^{Ex}*Taq* polymerase (TaKaRa), and filled up to a volume of 25 µl with sterile Milli-Q water. The thermal program consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94, 54 and 72°C for 30 s each, in which the annealing temperature is 72°C for 7 min (Ledder et al., 2007). Amplification products were analyzed first by electrophoresis of 1% agarose gel containing ethidium bromide, and compared to a molecular weight standard (100 bp).

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE were performed using D-Code™ Universal Mutation Detection System (Bio-Rad, Hercules, CA). The PCR products were electrophoresed on 8% polyacrylamide (acylamide/bisacrylamide, 37.5:1) gels containing a linear denaturant gradient ranging from 25 to 65%, with 100% denaturant defined as a solution of 7 M urea and 40% (*v/v*) deionized formamide. Electrophoresis was performed, first for 10 min at 200 V, and subsequently for 16 h at 70 V in a 1×TAE buffer at a constant temperature of 60°C. Gels were stained with $AgNO_3$ (Edenborn and Sexstone, 2007).

Stained gels were analyzed by using Quantity One 4.6.2 gel analysis software (Bio-Rad). Similarities were displayed graphically as a dendrogram. The clustering algorithms used to calculate the dendrograms were an unweighted pair group method with arithmetic average (UPGMA) (Du et al., 2006).

The Shannon-Wiener index of diversity (*H'*) (Gafan et al., 2005) was used to determine the diversity of the bacterial community. This index was calculated by:

$$H' = - \sum (p_i)(\ln p_i)$$

Where, p_i was the proportion of the bands in the track and was calculated as follows:

$$p_i = n_i / \sum n_i$$

Where, n_i was the average density of peak *i* in the densitometric curve.

Sequence analysis

The selected dominant bands were excised from the gel and eluted in 20 µl sterile water at 4°C overnight. 3 µl of the eluted DNA was reamplified by PCR following the program described above; only the forward primer was 357f without GC clamp. Each PCR product was also subjected to DGGE analysis to confirm whether the band was purified or not. Subsequently, idiographic sequences were attained by TaKaRa Biotechnology (Dalian) Co., Ltd. Finally, sequences were manually aligned with GenBank (NCBI).

RESULTS

Denaturing gradient gel electrophoresis (DGGE) analysis

In DGGE profiles of LH group, the amount and intensity of bands for different drug administration periods (Normal, I, II, and III) were different (Figure 1). Bacteria, *C*, *E* and *H* were remarkably increased in group I, II and III, but *B* was remarkably reduced or even extinguished in these drug administration groups, *D* was increased in group III, *F* and *G* had no significant change before and after the drug administration.

The clustering analysis based on the values of Dice coefficients was visualized in an UPGMA dendrogram to study general patterns of community similarity among the different administration periods of LH. A closer relationship existed between group normal and group III, another closer relationship existed between Groups I and II (Figure 2).

DGGE profiles displayed the typical characteristics of general bacteria in the intestinal tract. Each band derives possibly from one phylogenetically distinct community, hence, an estimation of species number could be based on the total number of the bands in the profile (Hu et al., 2007). The indices of *H'* reflecting the structural diversity of the bacterial community (Gafan et al., 2005), were calculated on the basis of the number and relative intensities of bands on the gel (Table 1).

The diversity of LH drug administration and drug stopping groups are all higher than the normal group in Table 1. Dysbacteriosis of intestinal microflora was induced by LH, possibly.

Sequence analysis of selected dominant bands of DGGE

Data in Table 2 shows the closest relatives based on results of BLAST searches with DNA sequences obtained from DGGE gel bands identified by cluster analysis.

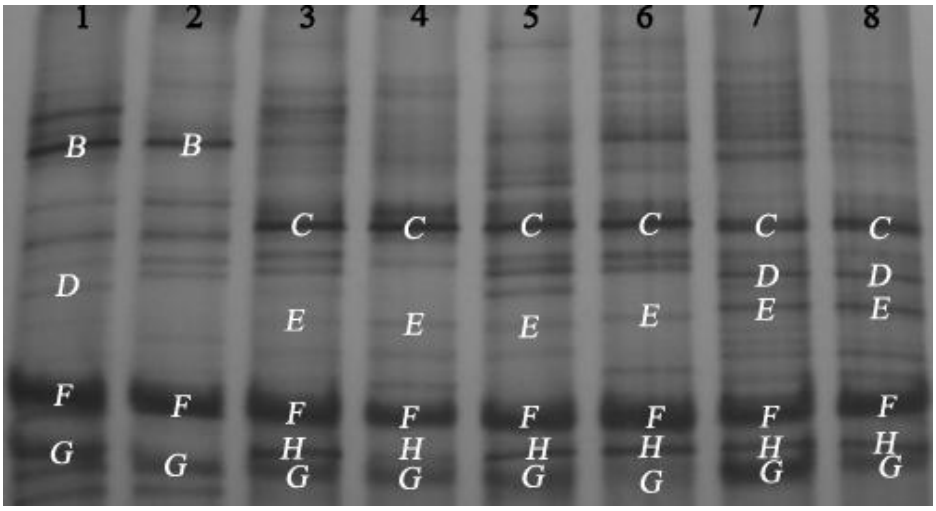

Figure 1. Representative DGGE profiles of different administration periods of LH groups. 1~2: Normal; 3~4: Given LH for 3d (I); 5~6: Given LH for 10d (II); 7~8: Stopping drugs for 7d (III).

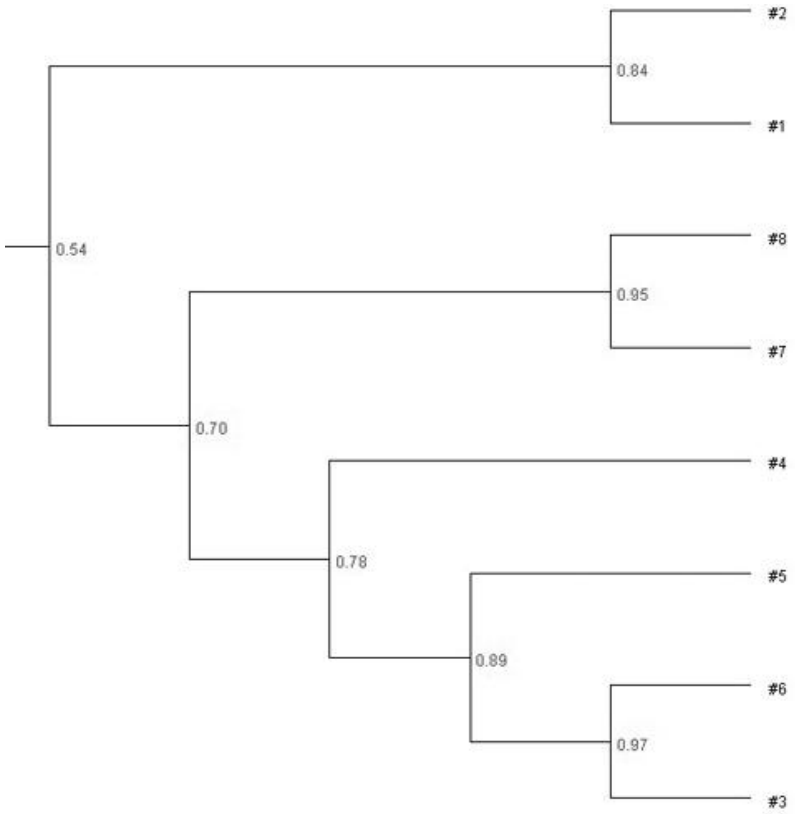

Figure 2. UPGMA dendrograms showing the percent DGGE profiles matching of different administration periods of LH groups. 1~2: Normal; 3~4: Given LH for 3d (I); 5~6: Given LH for 10d (II); 7~8: Stopping drugs for 7d (III).

Bands in the same position but in different lanes were excised and sequenced to confirm that they had the same identity (data not shown). The identities of *B* and *Prevotella amni*, *C* and *Porphyromonas uenonis*, *D* and *Bacteroides coprophilus*, *E* and *Streptococus sanguinis*, *F* and *Bacteroides sp.*, *G* and *Clostridium butyricum str.*,

Table 1. Shannon-Wiener index of diversity (*H'*) indexes calculated from the digitized DGGE patterns of LH groups[1] ($\bar{x}$ ±s, *n* = 10).

Diversity index	Normal	I	II	III
H'	0.4827±0.0011	0.6240±0.0006**	0.6226±0.0008**	0.5589±0.0001**

[1]Compare to normal group, respectively, **P<0.01.

Table 2. Sequences of PCR amplicons derived from DGGE gels and identities based on the BLAST database.

Selected band	V3 fragments (bp)	Most similar sequence relative (GenBank accession number)	Identities (%)
B	158	*Prevotella amnii* (ADFQ01000002.1)	97
C	158	*Porphyromonas uenonis* (ACLR01000152.1)	97
D	158	*Bacteroides coprophilus* (ACBW01000012.1)	96
E	153	*Streptococus sanguinis* (AFQB01000012.1)	97
F	150	*Bacteroides sp.* (ACTC01000133.1)	96
G	138	*Clostridium butyricum str.* (ACOM01000003.1)	97
H	157	*Leptotrichia hofstadii* (ACVB02000014.1)	83

bacteria *H* and *Leptotrichia hofstadii* were 97, 97, 96, 97, 96, 97 and 83%, respectively. In experiments of LH, *Porphyromonas uenonis*, *S. sanguinis* and *L. hofstadii* were identified in drug administration group (I, II) and even in drug stopping group (III); *Bacteroides coprophilus* increased remarkably in group III; *Bacteroides* and *C. butyricum* had no significant change.

DISCUSSION

All drugs come with adverse effects and antibiotics are no exception. Common side-effects are gastrointestinal symptoms, skin rashes and thrush, specific effects include nephrotoxicity associated with aminoglycosides and staining of the teeth attributable to tetracyclines (Dancer, 2004).

Fluoroquinolones, the most frequently reported adverse events associated with gastrointestinal and central nervous system reactions (Lipsky and Baker, 1998; Fish, 2001; Rodvold, 1999). These adverse events rarely require the discontinuation of therapy. Levofloxacin, a recent fluoroquinolone has already been involved in a case of acute rhabdomyolysis (Nakamae et al., 2000), and superinfection attributable to intestinal dysbacteriosis are also serious (Rafii et al., 2008).

DGGE analysis indicated that LH increased the total number of DNA, the bacterial composition and structure could be divided into different clusters for different drug administration periods distinctly, and sequence analysis indicated that *Prevotella amni*, *Porphyromonas uenonis*, *Bacteroides coprophilus*, *S. sanguinis*, *Bacteroides sp.*, *C. butyricum str.* and *L. hofstadii* were dominant organisms. Presumably, the transformation of dominant organisms could be the risk factors associated with side effects.

Porphyromonas is inhabitant of the intestinal microflora (Whitehead, 1997). It possesses a number of virulence factors, such as fimbriae, lipopolysaccharide, and cysteine proteinases (Holt et al., 1999), certain species are capable of causing disease states in humans. *S. sanguinis* is a member of the viridans group of streptococci (Swenson and Rubin, 1982). It has been reported to be closely related to infective endocarditis, a serious heart disease that can possibly lead to death (Douglas et al., 1993; Freedman, 1987). *L. hofstadii* is also normal microflora in the intestinal tract, not pathogenic generally, but it grows and causes gastrointestinal inflammation if the pH value of gastrointestinal tract changes. All above bacteria were increased in drug administration groups and did not exist in normal groups, but the difference was they still existed even after stopping drug administration, so side effects of LH on the intestinal tract cannot disappear immediately after stopping drug.

These three kinds of bacteria are all mainly opportunistic infection microbes of the intestinal tract, so increase of these resulted from LH is a main factor associated with side effects of the intestinal tract, which meanwhile even lead to superinfection. In addition, *P. amnii* was identified in normal mice, and decrease remarkably in LH drug administration and drug stopping groups. But on the contrary, *Bacteroides coprophilus* was increased in drug stopping group. As reported by Ley et al. (2005) and Lesley et al. (2009), *Prevotella* and *Bacteroides* were significantly associated with obesity, and dysbacteriosis symptoms will occur if their content changed in the intestinal tract, and hence disease will follow.

Bacteroides and *C. butyricum* are probiotics, they were antagonistic to pathogenic microorganisms of intestinal microflora (Kim et al., 1988; Murray et al., 1984), could help host to decompose polysaccharide and improve the

efficiency of nutrition (Bäckhed et al., 2004), speed up the vascularization of the gut mucosa (Stappenbeck et al., 2002), maintain the balance of intestinal microbiota (Sears et al., 2005; Hooper et al., 2001), modulate the gastrointestinal microflora (Kong et al., 2011). Fortunately, they were no significant changes before and after the drug administration, so dysbacteriosis can be improved slightly after stopping LH, but can't be healed due to the serious adverse effects of *Helicobacter pylori*, *Porphyromonas*, *S. sanguinis* and *L. hofstadii*.

In this study, the similarity, diversity, and sequence analysis of the dominant bands for different LH drug administration periods were carried out by DGGE. *Porphyromonas*, *S. sanguinis* and *L. hofstadii* have important adverse effects on the intestinal tract, increasing along with drug administration of LH, most seriously, they still existed even after the drug was stopped. It is because of the increase of the above three kinds of bacteria: it is the main factor associated with side effect of the intestinal tract that may even lead to superinfection, possibly.

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