Side effects of antibiotics on the intestinal microflora by PCR-DGGE

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Abstract: Antibiotics are recommended for use in the treatment of infectious diseases, but the side effects have not been thoroughly investigated, especially on the intestinal tract. Fluoroquinolones, macrolides and β-lactams were tested the side effects on the intestinal microbflora of mice in this study. The similarity 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 increased after drug administration, the bacterial composition and structure could be divided into different clusters for different drug administration periods distinctly. This study revealed a significant change of bacterial composition of microflora from intestinal tract as antibiotics were applied to tested mice.

Keywords: Antibiotics; Side effects; Intestinal microflora; PCR-DGGE

INTRODUCTION

Fluoroquinolones, macrolides and β-lactams are widely used for oral administration. Levofloxacin (LEV), roxithromycin (ROX) and cefradine (CEF) are representative of above three kinds of antibiotics, respectively. They provided a broad-spectrum antibiotic activity against both Gram-positive and Gram-negative pathogens (Karlowsky *et al*., 2003; Jones *et al*., 2003; Critchley *et al*., 2002; Jones *et al*., 1999; Flynn *et al*., 1996), but side effects accompanying the drug administration, especially intestinal tract, such as abdominal pain, diarrhea, astriction (Sprandel *et al*., 2003; Gisbert *et al*., 2008; Li *et al*., 2010; Reisner 1996).

DGGE based on sequence variability in 16S rRNA genes has been used. This molecular technique has been applied to identify the sequence variations in a number of genes from different organisms successfully (Muyzer *et al*., 1993). The advantage of this technique is DNA fragments of the same length but with different base-pair sequence could be separated (Fischer *et al*., 1979). The objective of this study was to investigate the side effects of fluoroquinolones, macrolides and β-lactams on the intestinal microflora of mice by PCR-DGGE. The similarity and sequence analysis of the dominant bands for different drug administration periods were carried out. The investigation of intestinal microflora affected by antibiotics would explore the risk factors associated with side effects of intestinal tract.

MATERIALS AND METHODS

Subjects and preparation of samples

Thirty female BALB/c mice aged 6 weeks of SPF grade were supplied by Animal Lab Center of Dalian Medical University, certificate of quality number is SCXK (Liao) 2008-0002. They were fed on normal diet and divided into three groups: LEV, ROX and CEF group, randomly, and each group included ten mice.

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, LEV was given at 65mg/kg for 10 days, and stopping drug for 7 days. As parallel, the dosage of ROX and CEF were 50mg/kg and 165 mg/kg, respectively.

Fecal samples were collected from per mice in each group at 0d (Normal), 3d (I), 10d (II) of drug administration periods, and stopping drug 7d (III) period, respectively.

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.

PCR amplification

Primers GC-341f (5'-**CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGACGGGGGG** CCTACGGGAGGCAGCAG), an additional 40-nucleotide GC-rich sequence ended at its 5', and 518r (5'-ATTACCGCGGCTGCTGG) (Muyzer *et al*., 1993) which were used to amplify the V3 region of bacterial 16S rDNA (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: 3μl purified genomic DNA as template, 10×^{Ex}Taq buffer (Mg^{2+} plus) 2.5μl, dNTP mixture 4μl, BSA (1mg/mL) 2.5μl, 10pmol of each primer, 1.25U of ^{Ex}*Taq* polymerase (TaKaRa), and filled up to a volume of 25μl with sterile Milli-Q water. The thermal program consisted of an initial denaturation step of 94°C for 5min, followed by 30 cycles of 94°C, 54°C, 72°C for 30s each, in which the annealing temperature of 72°C for 7min (Ledder *et al*., 2007).

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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 7M urea and 40% (*v/v*) deionized formamide. Electrophoresis was performed, first for 10min at 200V, and subsequently for 16h at 70V in a 1×TAE buffer at a constant temperature of 60°C. Gels were stained with $AgNO_3$ (Edenborn *et al*., 2007).

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

Sequence analysis

The selected dominant bands were excised from the gel and eluted in 20 µl sterile water at 4°C overnight. 3µl of the eluted DNA was reamplified by PCR following the program described above, only the forward primer was 341f without GC clamp. Each PCR product was also subjected to DGGE analysis to confirm the band purified or not. Subsequently, idiographic sequences were attained by TaKaRa Biotechnology (Dalian) Co., Ltd. Finally, sequences were manually aligned with GenBank (NCBI).

RESULTS

DGGE analysis

In DGGE profiles of LEV, ROX and CEF groups, the amount and intensity of bands of different drug administration periods (Normal, I, II, and III) were different (fig. 1). Bacteria *B*, *D* and *F* increased remarkably in I, II and III of LEV group, but *A* reduced or even extinguished, *C* was increased in group III, *E* and *G* were no significant change before and after the drug administration. There are some similarities between CEF and LEV groups. Particularly, Bacteria *B* increased, and *E* reduced remarkably in ROX administration group.

Clustering analysis which 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 the three kinds of antibiotics. A closer relationship existed between group normal and group III, another closer relationship existed between group I and group II (fig. 2).

DGGE profiles displayed the typical characteristics of general bacteria in the intestinal tract. Each band derives

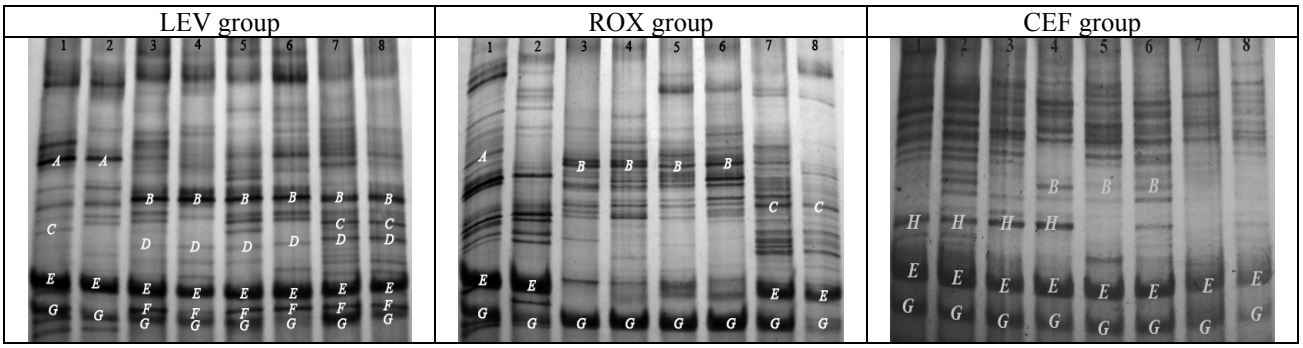

Fig. 1: Representative DGGE profiles of different administration periods
1~2: Normal; 3~4: Given drugs for 3d (I); 5~6: Given drugs for 10d (II); 7~8: Stopping drugs for 7d (III)

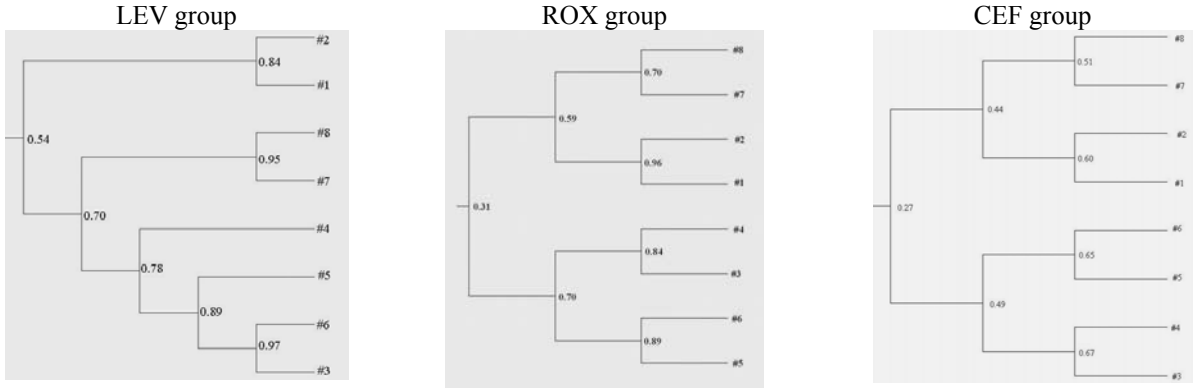

Fig. 2: UPGMA dendrograms showing the percent DGGE profiles matching of different administration periods.
1~2: Normal; 3~4: Given drugs for 3d (I); 5~6: Given drugs for 10d (II); 7~8: Stopping drugs for 7d (III)

Table 1: 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 (%)
A	158	*Prevotella amnii* (ADFQ01000002.1)	97
B	158	*Porphyromonas uenonis* (ACLR01000152.1)	97
C	158	*Bacteroides coprophilus* (ACBW01000012.1)	96
D	153	*Streptococus sanguinis* (AFQB01000012.1)	97
E	150	*Bacteroides sp.* (ACTC01000133.1)	96
F	157	*Leptotrichia hofstadii* (ACVB02000014.1)	83
G	138	*Clostridium butyricum str.* (ACOM01000003.1)	97
H	135	*Clostridium carboxidivorans P7* (ACVI01000229.1)	93

possibly from one phylogenetically distinct community, hence, an estimation of species number could be based on the total number of bands in the profile (Hu *et al*., 2007).

Sequence analysis of selected dominant bands of DGGE

Table 1 show the closest relatives which based on the results of BLAST with DNA sequences obtained from DGGE gel bands identified by clustering analysis. 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 *A* and *Prevotella amni*, *B* and *Porphyromonas uenonis*, *C* and *Bacteroides coprophilus*, *D* and *Streptococus sanguinis*, *E* and *Bacteroides sp.*, *F* and *Leptotrichia hofstadii*, *G*, *Clostridium butyricum str.*, and *H* and *Clostridium carboxidivorans P7* were 97%, 97%, 96%, 97%, 96%, 83%, 97% and 93%, respectively. In experiments of Levofloxacin, *Porphyromonas uenonis*, *Streptococus sanguinis* and *Leptotrichia 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 *Clostridium butyricum* were no significant change. And in the experiments of cefradine, *Porphyromonas uenonis* increased in group I and II, *Clostridium carboxidivorans P7* were identified in group normal and I, *Bacteroides* and *Clostridium butyricum* were no significant change, it is similar to Levofloxacin's. Particularly, in the experiments of roxithromycin, *Bacteroides* disappeared in drug administration group.

DISCUSSION

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

DGGE analysis indicated that the bacterial composition and structure could be divided into different clusters for different drug administration periods of fluoroquinolones, macrolides and β-lactams, distinctly. Sequence analysis indicated that *Prevotella amni*, *Porphyromonas uenonis*, *Bacteroides coprophilus*, *Streptococus sanguinis*, *Bacteroides sp.*, *Clostridium butyricum str.*, *Leptotrichia hofstadii* and *Clostridium carboxidivorans P7* 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 some virulence factors, such as lipopolysaccharide, fimbriae, and cysteine proteinases (Holt *et al*., 1999), certain species are able to cause disease in humans. *Streptococcus sanguinis* is a member of streptococci (Swenson *et al*., 1982). It has been closely related to infective endocarditis, a serious heart disease that can possibly lead to death (Douglas *et al*., 1993; Freedman, 1987). *Leptotrichia 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 was changed. All above bacteria were increased in fluoroquinolones administration groups and not exist in normal groups. These three kinds of bacteria are all mainly opportunistic infection microbes in the intestinal tract, so the increase of them resulted from fluoroquinolones is the main factor associated with side effects of the intestinal tract, meanwhile even lead to superinfection.

Bacteroides and *Clostridium 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). The difference between macrolides and fluoroquinolones is that *Bacteroides* disappeared in macrolides administration and unchanged before and after fluoroquinolones administration. The conditions of β-

lactams are similar to fluoroquinolones', the difference was *Porphyromonas uenonis* still existed even if stopping fluoroquinolones, so side effects of fluoroquinolones on the intestinal tract can't disappear immediately after stopping drug.

In addition, *Prevotella amnii* was identified in normal mice, *Bacteroides coprophilus* was increased in drug stopping group. As reported (Ley *et al*., 2005; Lesley *et al*., 2009), *Prevotella* and *Bacteroides* were significantly associated with obesity, and dysbacteriosis symptoms will occur if the content of them were changed in the intestinal tract, then diseases will follow.

In this study, the similarity and sequence analysis of the dominant bands for different drug administration periods of three kinds of antibiotics were carried out by DGGE. The increase of pathogenic bacteria just as *Porphyromonas uenonis*, *Streptococcus sanguinis*, *Leptotrichia hofstadii* and the decrease of probiotics just as *Bacteroides sp.* have important adverse effects on the intestinal tract. So selecting antibiotics, it should be taken into consideration that the side effects on the intestinal tract.

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