

Full Length Research Paper

Detection of intestinal microflora from mice affected by macrolide antibiotics

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Macrolide antibiotics are widely used for treatment of community-acquired infectious diseases, but its side effects have not been thoroughly investigated, especially on the intestinal tract. Erythromycin, roxithromycin and azithromycin are macrolide antibiotics sharing similar chemical structure and their side effects on intestinal microflora of BALB/c mice were tested in this study. The bacterial composition of microflora was determined by 16S rRNA gene analysis conducted by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). The dominant bands were selected and sequenced to determine each individual bacteria. The total amount of 16S rRNA gene was reduced after macrolide antibiotics were administrated, and the specific pattern of bacterial composition was identified from each drug treatment. *Bacteroides* sp. and *Clostridium butyricum* str. were dominant intestinal organisms in all three drug-treated mice. This study reveals a significant change of bacterial composition of microflora on the tested mice for macrolide antibiotics.

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

INTRODUCTION

The macrolide antibiotics were used to treat various infectious diseases for over 60 years. They were effective against pneumococcal, streptococcal and mycoplasmal infections (Retsema et al., 2001) particularly used for patients who are allergic to penicillins.

Macrolides were characterized for their molecular structure, a macrocyclic lactone ring attached to various amino sugars. The drug was classified according to the number of carbon atoms in the macrocyclic ring. The most important macrolide antibiotics are 14-, 15- and 16-membered-ring compounds which were subdivided into two major groups: natural products and semisynthetic derivatives (Bryskier, 1999). The first commercially available macrolides was erythromycin which was natural product and utilized as antibacterial agents in the treatment of community-acquired infectious diseases. Those semisynthetic compounds were further divided into three subgroups according to the types of chemical

modification on the core erythromycin structure. The first subgroup comprises those compounds with substituent modifications, such as roxithromycin, clarithromycin and flurithromycin. Compounds obtained from modifications of the aglycone, such as azithromycin, belong to the second subgroup. The compounds in the third subgroup including the ketolides are obtained by modification of the C-3 α -L-cladinose (Retsema et al., 2001). Although the new generation of macrolides provided a broad-spectrum antibiotic activity against both Gram-positive and Gram-negative pathogens (Peters et al., 1992), but side effects accompanying the drug administration are, such as abdominal pain, diarrhea, astriction, and etc (Reisner 1996).

Denaturing gradient gel electrophoresis (DGGE) based on sequence variability in 16S rRNA genes was successfully applied to identifying sequence variations in a number of genes from several different organisms

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(Muyzer et al., 1993); DNA fragments of the same length but with different base-pair sequence can be separated (Fischer et al., 1979). Erythromycin (ERY), roxithromycin (ROX) and azithromycin (AZI) were common macrolides widely used in clinic and shared similar chemical structure. The objective of this study was to investigate the effects of the three antibiotics on the intestinal microflora from BALB/c mice by PCR-DGGE. Bands from mice at different drug administration periods were analyzed to measure the similarity and diversity among the treatment groups, and the sequences of the dominant bands were determined. The investigation of intestinal microbiota affected by macrolides would explore the risk factors associated with side effects of the intestinal tract.

MATERIALS AND METHODS

Subjects and preparation of samples

Thirty (30) female BALB/c mice aged 6 weeks of specific pathogen-free (SPF) grade were supplied by Animal Lab Center of Dalian Medical University; certificate of quality number was SCXK (Liao) 2008-0002. They were fed on normal diet and divided into three groups: ERY, ROX, and AZI group randomly, and each group included ten mice.

Dosage (d) was according to the Meeh-Rubner conversion formula between human and mouse:

$$d_{\text{mouse}} (\text{mg/kg}) = d_{\text{human}} (\text{mg/kg}) \times (K_{\text{mouse}} / K_{\text{human}})$$

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

So, ERY group was administrated 330 mg/kg for 10 days, followed by no-drug condition for 7 days. As parallel, the dosage of ROX and AZI were 50 and 165 mg/kg, respectively.

Fecal samples were collected per mice in each group at 0 day (Normal), 3 days (I), 10 days (II) of drug administration periods, and no-drug 7 days (III) period, respectively, and stored at -80°C .

DNA extraction

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

PCR amplification

Primers GC-357f (5'-CGCCCCGGGCGCGCCCCGGGCGGGGCGGGGGCACGGG GGGCCTACGGGAGGCAGCAG), 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, 2.5 μl $10\times$ ExTaq buffer (Mg^{2+} plus), 4 μl dNTP mixture, 2.5 μl BSA (1 mg/mL), 10 pmol of each primer, 1.25 U of ExTaq polymerase (TaKaRa), which was 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, with the annealing temperature of 72°C for 7 min (Ledder et al., 2007). Amplification products were analyzed first by electrophoresis of 1% agarose gel containing ethidium bromide, and comparison to a molecular weight standard (100 bp).

DGGE analysis

DGGE were performed using D-Code[™] Universal Mutation Detection System (Bio-Rad, Hercules, CA). The PCR products were electrophoresed on 8% polyacrylamide (acrylamide: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 \times 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 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 by following the program described previously; only the forward primer was 357f without GC clamp. Each PCR product was also subjected to DGGE analysis to confirm where the band was purified or not. Subsequently, idiographic sequences were attained by TaKaRa Biotechnology (Dalian) Co., Ltd. Finally, sequences were manually aligned with GenBank.

RESULTS

DGGE analysis

DGGE profiles of three macrolide antibiotics, ERY, ROX, and AZI groups were similar, but the amount and intensity of bands from different drug administration periods (Normal, I, II, III) in the same macrolides were different (Figure 1). A was remarkably reduced or even extinguished in groups I and II, and the effect of AZI on A was smallest among the three macrolides; the effect of ERY was greatest. B had no significant change before and after the drug administration.

The clustering analysis based on the values of Dice

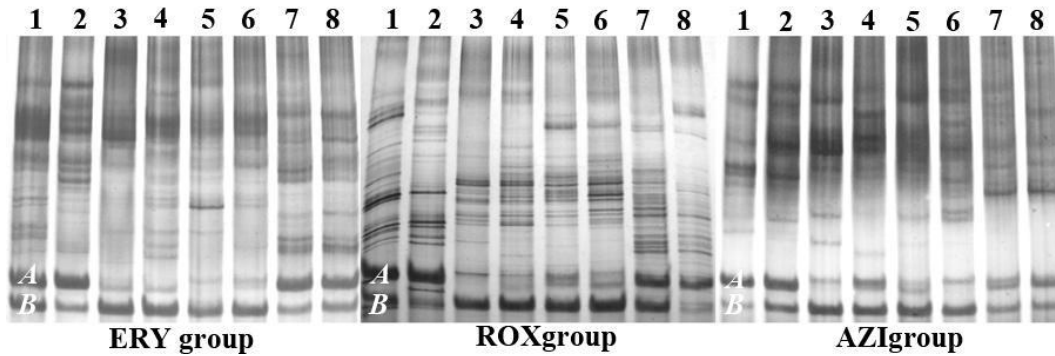


Figure 1. Representative DGGE profiles of different administration periods of ERY, ROX, and AZI groups. 1-2, Normal; 3-4, given macrolides for 3 days (I); 5-6, given macrolides for 10 days (II); 7-8, No-drugs for 7 days (III).

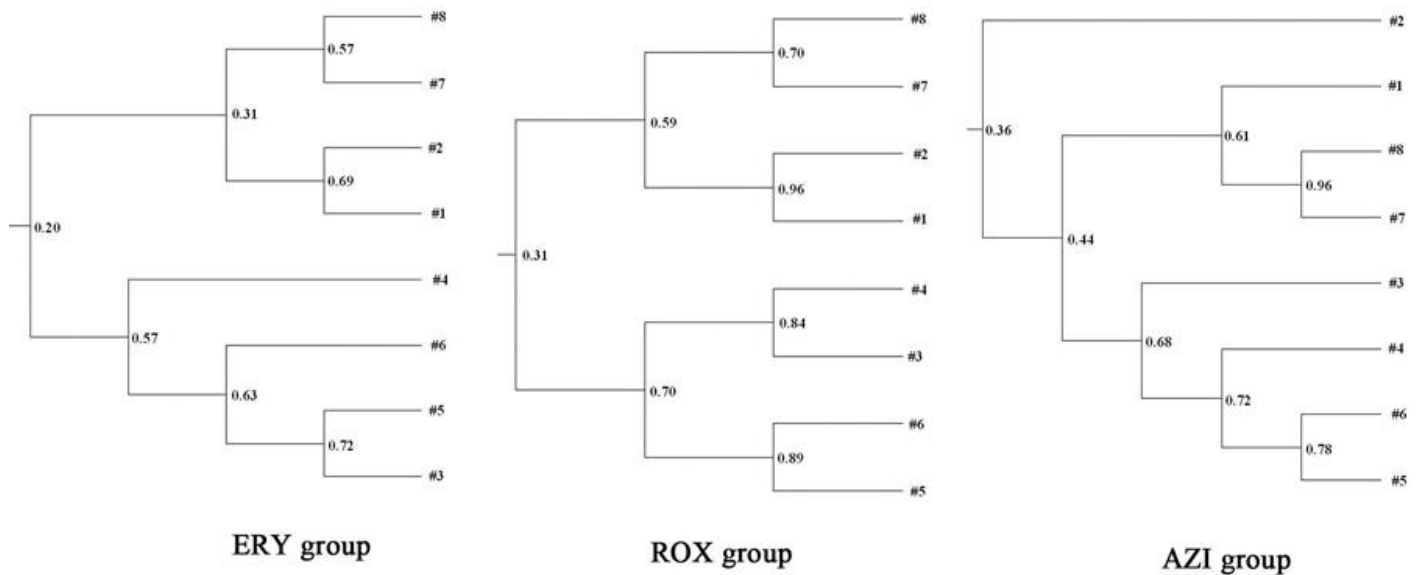


Figure 2. UPGMA dendrograms showing the percent DGGE profiles matching of different administration periods of ERY, ROX, and AZI groups. 1-2, Normal; 3-4, given macrolides for 3 days (I); 5-6, given macrolides for 10 days (II); 7-8, No-drugs for 7 days (III).

coefficients was visualized in an UPGMA dendrogram to study the general patterns of community similarity among the different administration periods of ERY, ROX, and AZI. A closer relationship existed between the normal group and group III, and another closer relationship existed between group I and group II (Figure 2).

DGGE profiles displayed the typical characteristics of general bacteria in the intestinal tract. Each band was derived 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 macrolides administration group was lower than that of the normal group from Table 1. Dysbacteriosis of intestinal microflora was possibly induced by macrolide antibiotics.

Sequence analysis of selected dominant bands of DGGE

Table 2 shows the closest relatives based on results of BLAST searches with DNA sequences obtained from DGGE gel bands identified by cluster 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 *Bacteroides* sp.,

Table 1. Shannon-Wiener index of diversity (H') index calculated from the digitized DGGE patterns of of ERY, ROX, and AZI groups¹ ($\bar{x} \pm s$, $n = 10$).

Number	H'	Number	H'	Number	H'
ERY-normal	2.4827±0.0004	ROX-normal	0.4571±0.0008	AZI-normal	0.3731±0.0340
ERY-I	1.0968±0.0002**	ROX-I	0.5615±0.0003**	AZI-I	0.4830±0.0010*
ERY-II	1.2415±0.2032*	ROX-II	0.5004±0.0236	AZI-II	0.4503±0.0462
ERY-III	2.1378±0.0831	ROX-III	0.4878±0.0433	AZI-III	0.5584±0.0002

¹Each macrolide antibiotic compared to its own normal group, respectively. ** $p < 0.01$, * $p < 0.05$.

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

Band number	V3 fragment (bp)	Most similar sequence relative (GenBank accession number)	Identity (%)
A	150	<i>Bacteroides</i> sp. (ACTC01000133.1)	96
B	138	<i>Clostridium butyricum</i> str. (ACOM01000003.1)	97

B and *Clostridium butyricum* str. were 96 and 97%, respectively. In the experiments of three macrolides, *Bacteroides* sp. were identified in the normal group and the drug stopping group (III), but not in the drug administration groups (I and II). *C. butyricum* str. had no significant change.

DISCUSSION

The macrolides remain a cornerstone of antibacterial therapy. They are extensively prescribed for the treatment of upper and lower respiratory tract infections of suspected bacterial aetiology (Carbon et al., 1999). It is reported that the stimulation of gastrointestinal motility is a common side effect of macrolides (Williams, 2001), and they also lead to gastrointestinal disorders (Smith et al., 2005).

The total number of DNA was reduced after treatment with each of the three drugs based on DGGE analysis, but the bacterial composition were divided into different clusters in all different drug administration periods. *Bacteroides* sp. and *C. butyricum* str. were dominant organisms according to sequence analysis. Presumably, the alternation of dominant organisms may be one of the risk factors associated with side effects.

Bacteroides sp. contributed to human health by decomposing polysaccharide and improving the efficiency of nutrition (Bäckhed et al., 2004) as well as increasing the vascularization of the gut mucosa (Stappenbeck et al., 2002), and maintaining the balance of intestinal microflora (Sears, 2005; Hooper et al., 2001). The decrease of *Bacteroides* sp. caused by macrolide antibiotics was the

main factor leading to dysbacteriosis. Meanwhile, with the development of new macrolide antibiotics, the adverse effects on *Bacteroides* sp. were gradually decreased from earlier ERY to latest ROX and AZI suggesting that the modification of the core structure not only provide an extended spectrum of antibiotic activity, but also decrease the intestinal side effects.

C. butyricum str. were Gram-positive spore-forming anaerobic bacteria and resistant to multiple antibiotics. The bacterium were also capable to promote the development and proliferation of intestinal probiotics, restrain the growth and reproduction of harmful bacteria, correct intestinal disorder, and reduce the occurrence of intestinal toxins. The microecologies of *C. butyricum* str. were used to treat the diarrhea caused by pathogen, irritable bowel syndrome diarrhea and antibiotic-associated diarrhea (Kim et al., 1988). In this study, *C. butyricum* str. had no significant change after the drug administration, therefore it was postulated that *C. butyricum* str. may play an important role on natural recovery of dysbacteriosis symptoms caused by macrolides since the growth of probiotics might be boosted by *C. butyricum* str. (Kong et al., 2011).

In this study, the similarity, diversity, and sequence analysis of the dominant bands for different macrolide antibiotics drug administration periods were carried out by DGGE. *Bacteroides* sp. decreased along with drug administration. It is the main factor associated with the side effect of the intestinal tract that may even lead to superinfection, possibly. According to our another study on the side effects of Levofloxacin hydrochloride, *Porphyromonas*, *Streptococcus sanguinis* and *Leptotrichia hofstadii* which still existed even after drug

stopped are the main factors (Li et al., 2012). So, effects of different kinds of antibiotics on the intestinal microflora are different, presumably, and whether it is associated with the mechanism of antibiotic or not is not clear. Some further studies will be carried out in the future.

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