Microbiological Research 206 (2018) 9–15

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Combination of amplified rDNA restriction analysis and high-throughput sequencing revealed the negative effect of colistin sulfate on the diversity of soil microorganisms

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ARTICLE INFO

Keywords:
Colistin sulfate
Veterinary medicine
Ecological toxicity
Microbial diversity

ABSTRACT

Colistin sulfate is widely used in both human and veterinary medicine. However, its effect on the microbial ecologyis unknown. In this study, we determined the effect of colistin sulfate on the diversity of soil microorganisms by amplified rDNA restriction analysis (ARDRA) and high-throughput sequencing.ARDRAshowed that the diversity of DNA from soil microorganisms was reduced after soil was treated with colistin sulfate, with the most dramatic reductionobserved after 35 days of treatment. High-throughput sequencing showed that the Chao1 and abundance-based coverage estimators (ACE) were reduced in the soils treated with colistin sulfate for 35 dayscompared to those treated with colistin sulfate for 7 days. Furthermore, Chao1 and ACE tended to be lower when higher concentration of colistin sulfate was used, suggesting that the microbial abundance is reduced by colistin sulfate in a dose-dependent manner. Shannon index showed that the diversity of soil microorganism was reduced upon treatment with colistin sulfate compared to the untreated control group. Following 7 days of treatment, *Bacillus*, *Clostridium*and *Sphingomonas* were sensitive to all the concentration of colistin sulfate used in this study. Following 35 days of treatment, the abundance of *Choroplast, Haliangium, Pseudomonas, Lactococcus, and Clostridium* was significantly decreased. Our results demonstrated that colistin sulfate especially at high concentration (≥5 mg/kg) could alter the population structure of microorganisms and consequently the microbial community function in soil.

1. Introduction

The residual antibiotics in the environment have increased with the development of livestock and aquaculture. The impact residual on ecosystems and human health has attracted more and more attention (Jechalke et al., 2014; Wohde et al., 2016; Santos et al., 2010; Boxall, 2010; Balakrishna et al., 2017; Kosma et al., 2014; Papageorgiou et al., 2016). Colistinsulfate is an antibiotic widely used in humans and animals for prevention and treatment of bacterial infections or to promote animal growth (Morales et al., 2012; Ordooei Javan et al., 2015; Karaiskos et al., 2017). Colistin sulfate is difficult to be absorbed after oral uptake, and therefore majority of colistin sulfate is secreted into the environment with the animal excrement (Rhouma et al., 2016; Cai et al., 2015; Rhouma et al., 2015; He et al., 2011). However, there was lack of research on the ecotoxicity of colistin sulfate, especially its ecotoxicity on the soil surroundingthe farms.

16S rDNA is well conserved and it accounts for approximately 80% of total bacterial RNA. Thus, it has been widely used to study the soil bacterial diversity (Loong et al., 2016; Benga et al., 2014; Woo et al., 2008). Amplified rDNA restriction analysis (ARDRA) is a technique that

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http://dx.doi.org/10.1016/j.micres.2017.09.002
Received 18 June 2017; Received in revised form 14 August 2017; Accepted 8 September 2017
Available online 14 September 2017
0944-5013/

analyzes the diversity of bacteria by digesting the amplified rDNA fragments and analyzing the restriction pattern (Sklarz et al., 2009; Santos et al., 2011; Öztürk and Meterelliyöz, 2015). Although ARDRA has high ability to differentiate bacterial species, it is relatively difficult for quantitative analysis and the comparisons between populations. High-throughput sequencing is a recently developed technique for studying microbial diversity (Tsilimigras and Fodor, 2016; Di Bella et al., 2013; Normand and Yanai, 2013; Ren et al., 2016). Usually, one or several hypervariable regions of 16SrDNA were amplified and then sequenced for identification of particular bacterial species. This method has become an important means to analyze the structure of microbial composition in environmental samples and can overcome the disadvantages of ARDRA. In this study, we combined ARDRA and high-throughput sequencing to explore the effect of colistin sulfate on soil bacteria, and to provide theoretical basis for the study of ecotoxicity of colistin sulfate.

2. Materials and methods

Collection and treatment for soils samples were collected from the experimental vegetable land (10cm–20 cm below the surface layer) at Guangdong Ocean University. Colistin sulfate was purchased from Shandong Lu Kang Pharmaceutical Co., Ltd. (batch number: 150092214). Fresh soil was filtered through a 4mm-sieve, and the physical and chemical properties were determined. The soil sample had a pH of 6.98, organic substance of 24.65 g/kg, total nitrogen of 2.41 g/kg, total phosphorus of 6.82 g/kg, total potassium of 11.47 g/kg, the available phosphorus of 11.58 mg/kg and the available potassium of 134 mg/kg. The soil samples were treated with 0 mg/kg, 0.5 mg/kg, 5 mg/kg, and 50 mg/kg colistin sulfate, respectively. Three replications were performed for each treatment. Soil moisture content was adjusted to 50%, which is the maximum water holding capacity in the field. The soil surface was covered with a damp cloth and placedat room temperature (20–25 ˚C) with the humidity of 70%-80. The soil sample was taken at 7d, 21d, 35d and 49d after treatment with colistin sulfate for ARDRA.

2.1. Soil bacteria culture

Soil samples (10 g) collected at different times were placed in sterilized conical flask, and 90 ml of sterilized water was added. The suspension was diluted 10^{-1} to 10^{-6} after shaking for 30 min. A total of 150 µl suspension from each dilution was evenly spread on LB agar and cultured for 24 h.

2.2. DNA template preparation

Bacteria colonies on the LB agar were inoculated into 2 ml of LB liquid medium, and cultured at 37 ˚C for 16 h with shaking at150 rpm. Following centrifugation at 12,000rmp for 2 min, the supernatant was discard and 1 ml sterile water was added. The mixture was centrifuged at 12,000rmpfor 2 min and supernatant was discarded. Finally, 200 µl sterile water was added and boiled at 100 ˚Cfor 10 min. Following immediate centrifugation, the supernatant was transferred to a new centrifuge tube and stored at-20 ˚C.

2.3. PCR amplification of 16S rDNA

The following universal primers for the 16S rDNA of prokaryotic organisms were used (Wang et al., 2010): F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and R: 5′-ACGGTTACCTTGTTACGACTT-3′. A total of 25 µl reaction including Taq polymerase 12.5 µl, primers 1 µl for each, DNA 2 µl and water 8.5 µl was set up with the reaction parameters of 94 ˚C 1 min, 45 cycles of 94 ˚C 1 min, 56 ˚C 30 s, and 72 ˚C 45 s, followed by 10 min extension for 10 min. PCR product (8 µl) was visualized after electrophoresis.

2.4. Digestion of the PCR product

Restriction enzymes MspI and RasI were used for the digestion of the PCR product with the following reaction: PCR product 15 µl, MspI 1 µl, RasI 1 µl and 10xbuffer 2 µl. After incubation at 37 ˚C for 3–5 h, electrophoresis at 1.8% agarose was performed before imaging.

2.5. Process of the restriction digestion data

After digestion and electrophoresis, the presence of a band was recorded as “1” and absence of a band was recorded as “0”. Using Quantity One, DNA with the same restriction ARDRA pattern was considered as the same genotype. Each genotype represented one Operational Taxonomic Units(OTU). The data was process with Excel and the average was calculated.

2.6. High-throughput sequencing of 16S rDNA

We performed high-throughput sequencing at two time points: 7 days and 35 days after the soil samples were treated with 0, 0.5, 5 and 50 mg/kg colistin sulfate. We chose 35 days becauseit resulted in maximum changes of the DNA bands in ARDRA. Total DNA was isolated at the two time points and PCR was performed using following primers to amplify the variable region of 16S rDNA. Primers 5′-CCTACGGRRBGCASCAGKVRVGAAT and 5′- GGACTACNVGGGTWTCTAATCC were used to amplify V3 V4 region. Primers 5′- GTGYCAGCMGCCGCGGTAA and 5′- CTTGTGCGGKCCCCGYCAATTC were used to amplify V4 V5 region. PCR products were sequenced by Genewiz using IlluminaMiSeq platform. Sequences were used for phylogenetic analysis using NTSYS, SPSS, Excel and R language. MEGA 5.0 was used for phylogenetic trees analysis and R language was used for heatmap.

3. Results

3.1. ARDRA analysis

The size of PCR product for 16S rDNA is approximately 1800 bp.

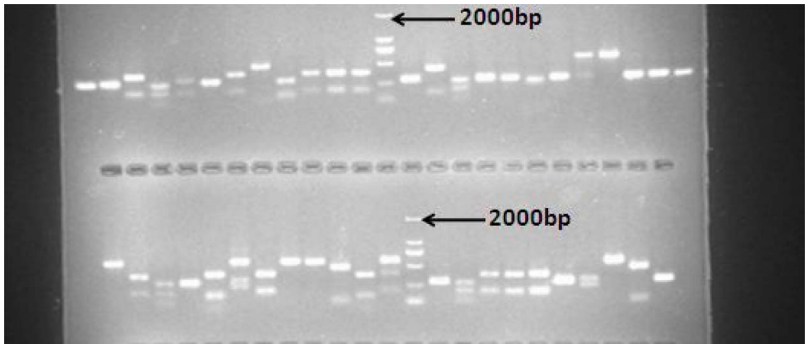

Fig. 1. Representative electrophoresis band patterns of 16SrDNA PCR fragment digested by MspI and RsaI. Each lane represents digestion of 16SrDNA PCR fragment amplified from DNA isolated from one bacterial sample.