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Research article

Evaluation of antibiotic resistant lactose fermentative opportunistic pathogenic *Enterobacteriaceae* bacteria and bla_{TEM-2} gene in cephalosporin wastewater and its discharge receiving river

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ABSTRACT

This study investigated the concentration of cephalosporin, the resistant levels of lactose fermentative opportunistic pathogenic *Enterobacteriaceae* bacteria (LFOPEB) against seven antibiotics and one cephalosporin-resistant gene in cephalosporin wastewater (CPWW) treatment plant and its discharge receiving river. Although large numbers of bacteria have been removed during the CPWW treatment process, the antibiotic resistant rates of the isolates to β-lactam antibiotics significantly increased ($p = 0.032$) after treatment, while the percentage of resistant LFOPEB to non-β-lactam antibiotics did not change dramatically. Furthermore, the discharge of the effluent of CPWW treatment plant ($CPWW_{eff}$) led to an obvious increase in the percentages of β-lactam antibiotic-resistant LFOPEB and relative abundance of the *blaTEM-2* gene in the downstream receiving river (RW_{down}) in comparison with those in the upstream receiving river (RW_{up}). The antibiotic resistant phenotypes of isolates in the influent of CPWW treatment plant ($CPWW_{in}$), $CPWW_{eff}$ and RW_{down} appeared to be seriously affected by the cephalosporin residues, which suggested that main antibiotic resistance phenotypes in antibiotic contaminated water were closely associated with its antibiotic composition. Therefore, CPWW treatment process has been proved to result in selective growth of ARB and proliferation of ARG. Besides, $CPWW_{eff}$ was also proved to be an important supplier of ARB and ARG to the receiving river.

1. Introduction

The prevalence of bacterial resistance to antibiotics has become an increasingly serious problem worldwide, which is rising a great threat to human health (Berglund et al., 2014; Pruden et al., 2013; Alderman and Hastings, 1998). In recent years, great numbers of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been extensively detected in various environmental media, such as sewage (Figueira et al., 2011; da Costa et al., 2006), lakes (Czekalski et al., 2012), rivers (Su et al., 2012), harbors (Lapara et al., 2011) and so on. Moreover, some scholars pointed out that antibiotics at sub-inhibitory concentrations could amplify the growth of ARB and accelerate the horizontal transfer frequency of ARGs (Bruchmann et al., 2013; Forsberg et al., 2012), which meant the microorganisms in the wastewater discharge receiving environment faced high risk of increasing antibiotic resistant level subsequently. Therefore, special attention has been paid to wastewater when researching the antibiotic resistant problem. (Michael et al., 2012; Picao et al., 2013).

Some surveys have confirmed the positive correlation between the frequency of ARB and concentration of antibiotic in antibiotic-contaminated places (Varela et al., 2014; Phan et al., 2011). Compared with other kinds of wastewater, antibiotic wastewater often contains

Abbreviation: Lactose fermentative opportunistic pathogenic *Enterobacteriaceae* bacteria, LFOPEB; Antibiotic-resistant bacteria, ARB; Antibiotic resistance genes, ARGs; Cephalosporin wastewater, CPWW; Effluent of cephalosporin wastewater treatment plant, CPWWeff; Influent of cephalosporin wastewater treatment plant, CPWWin; River water, RW; Downstream receiving river, RWdown; Upstream receiving river, RWup; Cefuroxime, CXM; Cefazolin, CFZ; Ceftriaxone, CTR; Cefotaxime, CTX; Levofloxacin, LVX; Trimethoprim, TMP; Ampicillin, AMP; Amoxicillin, AMC; Minimal inhibitory concentrations, MICs; Clinical and laboratory standards institute, CLSI; Multi-antibiotic resistant, MAR; Statistic package for social science, SPSS; Polymerase chain reaction, qPCR

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much higher concentrations of antibiotics coming from the upstream manufacture processes (Sim et al., 2011), which indicated the possibility of possessing higher percentage of ARB and larger abundance of ARG. Cephalosporin is a kind of β-lactam antibiotics with broad antimicrobial spectrum, especially for Gram-negative bacteria. It is one of the most widely used classes of antimicrobials in many countries (Harris et al., 2012; Versporten et al., 2012). However, there are limited researches on the antibiotic resistance problems resulting from antibiotic wastewater discharge (Liu et al., 2012; Li et al., 2009; Wang et al., 2015). Little is known about the characteristics of ARB and ARG in the antibiotic wastewater, especially in CWPP treatment plants.

Therefore, in this study, the antibiotic resistant levels of microorganisms in a CPWW treatment plant and its receiving river in a North China city were investigated. *Enterobacteriaceae* are one of the most widespread Gram-negative opportunistic pathogenic bacteria in nature. Thus, LFOPEB were chosen as the indicator microorganism in this study to analyze the presence of ARB in water samples. The hydrolysis of β-lactams by TEM β-lactamases encoded by bla_{TEM} gene is considered as the most common mechanism for bacteria to acquire resistance to β-lactam antibiotics (Mabilat and Courvalin, 1990). Therefore, we chose one kind of bla_{TEM} genes to represent the β-lactam antibiotic resistant level in genetic way.

To the best of our knowledge, it is the first systematic survey to investigate the impact of the CWPP treatment plants on the prevalence of antibiotic resistance problem. The objectives of this research were to obtain a general understanding to the multi-antibiotic resistant level of Gram-negative opportunistic pathogenic bacteria presented in CPWW, to investigate the influence of wastewater treatment process on antibiotic resistance, and to illustrate the impact of cephalosporin wastewater discharge on ARB and ARGs in the receiving river.

2. Methods and materials

2.1. Wastewater treatment plant and sample collection

The CWPP treatment plant was located in a pharmaceutical industrial park in a Northern city, China, which receives wastewater from a cephalosporin manufacture that has an annual production of 3000 t of cephalosporin. The main products of the upstream manufacture are cefuroxime (CXM), cefazolin (CFZ), ceftriaxone (CTR) and cefotaxime (CTX). The CPWW treatment plant uses the two stage tandem biological oxidation process, with the treatment capacity of 3000 m^3/d. The treated CPWW is discharged into a river for landscape use.

$CPWW_{inf}$ and $CPWW_{eff}$ samples were taken from the equalization tank and the outlet, respectively. Meanwhile, the receiving river samples were collected at the upstream 200 m (RW_{up}) and downstream 2000 m (RW_{down}) from the outfall, respectively. Both CPWW and RW samples were collected in the form of composite samples from three individual samples collected at 8 h intervals on 1st Sep and 25th Nov in 2014, and on 1st Mar and 29th May in 2015.

40 L water samples (10 L for each sample) were collected in sterilized glass bottles and transported to the laboratory in refrigerated box in dark within 4 h after collection. Experiments of total heterotrophic bacteria enumeration and total LFOPEB enumeration from water samples were carried out immediately at the arrival of samples to the laboratory. Analysis of the conventional water quality parameters were done in 24 h. All samples were preserved in a 4 °C freezer. Measurements of the physicochemical parameters were conducted according to APHA (2005) standard methods.

2.2. Quantification of cephalosporin

Concentrations of CXM, CFZ, CTR, and CTX in water samples were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (Quattro Premier XE, Waters). The reference compounds were purchased from Sigma-Aldrich, USA. High-purity water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase. The optimized system operating parameters for measuring the selected cephalosporin are shown in Table S1 in the supplementary material.

2.3. Enumeration of total heterotrophic bacteria and the isolation of LFOPEB

The enumeration of total heterotrophic bacteria was carried out based on plate count method. Wastewater and river water samples were firstly diluted with sterile deionized water so as to obtain 30–300 colony forming units per plate. Plates with 1 mL diluted water and 15 mL nutrient agar medium (peptone: 10 g/L, beef extract: 3 g/L, NaCl: 5 g/L, agar: 20 g) were inoculated at 37 °C for 24 h. Then the number of colonies were counted.

Diluted water samples (100 mL) were filtered through 0.45 μm membrane. The membrane was then incubated on fuchsin-basic sodium sulfite agar (Aoboxing, Beijing). After inoculated at 37 °C for 24 h, the red colonies with metallic sheen on the membrane were picked up and analyzed by Gram strain test. Gram-negative bacteria were then incubated in lactose peptone broth at 37 °C for 24 h for acid and gas production capacity test. Isolates that proved to produce acid and gas were further biochemically tested with API microbiological analysis system and API 20E kit (BioMerieux products, France) for strain identification according to the manufacturer's instructions. *Escherichia coli* ATCC 25922 (China center of industrial culture collection, Beijing) was used as a positive control.

2.4. Analysis of antibiotic resistant patterns among LFOPEB

Antibiotic resistant levels of identified LFOPEB against seven antibiotics were evaluated by using E-test strips (BioMerieux products, France) according to the manufacturer's instructions. Bacterial suspension of 0.5 McFarland turbidity standard was applied on Muller-Hinton agar. Plates were incubated at 35 °C for 18 h after E-test strips were placed. *E. coli* ATCC 25922 was used as quality control. The minimal inhibitory concentrations (MICs) were used to distinguish whether the isolated LFOPEB was resistant to the tested antibiotic. The MICs standard was as listed in CLSI (Clinical and Laboratory Standards Institute) documentation (Clinical and laboratory standards institute, 2016). Seven kinds of antibiotics were chosen, and their information (including MICs was displayed in Table 1. Strains that showed “intermediate” or “resistant” patterns subsumed under the “resistant” category, while the other strains that showed “sensitive” behavior were grouped in the “sensitive” category (Reinthaler et al., 2003). In each sampling point, percentage of LFOPEB that were resistant to a specific kind of tested antibiotic was defined as antibiotic resistant rate (r), which was calculated according to the following equation:

$$r = \frac{B_r}{B} \times 100\% \quad (1)$$

where B_r is the number of LFOPEB that shown resistant to the

Table 1
The properties of the antibiotics tested for resistance.

Antibiotics	Chemical group	Abbreviations	MICs interpretive criteria for resistant *Enterobacteriaceae* (μg/mL)
gentamicin	aminoglycoside	GTM	16
levofloxacin	fluoroquinolone	LVX	8
trimethoprim	dihydrofolate reductase inhibitors	TMP	16
ampicillin	β-lactam	AMP	32
amoxicillin	β-lactam	AMC	32
cefuroxime	β-lactam	CXM	32
ceftriaxone	β-lactam	CTR	4