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Occurrence and diversity of antibiotic resistance in untreated hospital wastewater

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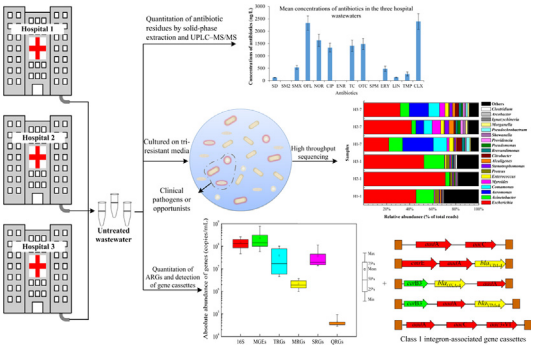
HIGHLIGHTS

- Antibiotic resistance was assessed in untreated hospital wastewater.
- High-frequency ARGs were detected with high-capacity qPCR.
- Some clinical pathogenic or opportunistic MARB were detected with high prevalence.
- Positive correlations between MGEs and some ARGs were detected.
- High concentrations of ARGs and MEGs were detected.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics, antibiotic-resistant bacteria (ARB), antibiotic-resistance genes (ARGs), and mobile genetic elements (MGEs) have been reported in many environments. However, the investigation of their occurrence and diversity in untreated hospital wastewater is still insufficient. High concentrations of antibiotic residues were found in hospital wastewater using solid-phase extraction and UPLC–MS/MS analysis. The concentrations of six of 14 antibiotics reached μg/L levels in the hospital wastewater, which is higher than reported in other aquatic environments. Results of high-throughput sequencing analysis indicated that sequences affiliated to genera *Escherichia* and *Acinetobacter* were the predominant in the cultivable multiple-antibiotic-resistant bacteria (CMARB) recovered from the wastewater of three hospitals in China, with compositions of 34%–74%. Notably, several genera containing clinically pathogenic or opportunistic CMARB (e.g., *Escherichia*, *Acinetobacter*, *Aeromonas*, *Myroides*, *Enterococcus*, *Proteus*, *Pseudomonas*, and *Streptococcus*) were detected at high relative abundances in the wastewaters of the three hospitals. High-capacity quantitative PCR showed that 131–139 unique ARGs of the 178 targeted genes were detected in the hospital wastewaters. The high prevalence of five MGEs and 12 ARGs was confirmed with qPCR, and some positive correlations between ARGs and MGEs were identified, such as between *intI1* and *qnrD*, *intI2* and *sul3*, *intI3* and *tetX*, Tn*916*/Tn*1545* and *sul2*, and IS*CR1* and *sul3*. These results suggest that highly abundant antibiotic-resistant pathogens and highly mobile ARGs already exist in the human body, and that their release from hospitals without effective treatment poses high risks to environments and human health.

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1. Introduction

Since their introduction into human medicines in the 1940s, antibiotics have been the most important drugs for the treatment of infectious diseases. However, antibiotic-resistant pathogens have emerged and spread among human and animal populations worldwide in response to the selection pressure exerted by the overuse of antibiotics. Some antibiotic-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (Grundmann et al., 2006) and carbapenem-resistant Enterobacteriaceae (Patrice et al., 2011), pose significant risks to human health. Hospital wastewater is highly hazardous because it is not only infectious and toxic, but is also an important source of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs). Without suitable treatment, ARB or ARGs from clinical sources can be dispersed and even thrive in the environments, accelerating the development of multiple-antibiotic-resistant bacteria (MARB).

With the increasing concerns about the antibiotic pollution of aquatic environments, several studies have assessed the presence of antibiotics, ARGs, and ARB in hospital effluents (Le et al., 2016; Rodríguez-Mozaz et al., 2015; Timraz et al., 2017; Varela et al., 2016), and other studies have examined the correlation between antibiotics and ARGs (Li et al., 2016) or between antibiotics and ARB (Varela et al., 2014). However, there have been far fewer studies of hospital aquatic ecosystems than of other environments, such as urban wastewater. Most reports focused on the influents or effluents from hospital treatment plants, in which the distribution and diversity of antibiotics, ARGs, and ARB were altered in response to the selective pressure imposed by long-term antibiotic exposure. Few reports were found to study the occurrence of antibiotic resistance in the untreated hospital wastewater, in which the antibiotics unlikely to affect ARB and ARGs. These wastewaters reflect the integrated discharges from humans, especially when patient density is high, and therefore reflect the original ARB and ARGs in human clinical samples. The human microflora, especially intestinal bacteria, is a reservoir for diverse ARGs. These bacteria not only exchange resistance genes among themselves, but can also interact with the bacteria that pass through the body, causing those bacteria to acquire and transmit ARGs (Sommer et al., 2010; Van den Braak et al., 1998). Furthermore, the ARB and ARGs that are released from the human body might then return to their original habitats (e.g., food, water, soil) and pose an increased environmental risk (Salyers et al., 2004). Therefore, investigation of antibiotic resistance in untreated hospital wastewater is helpful for understanding how clinical antibiotic resistance affects the environmental ARB.

Levels of antibiotic resistance in hospital wastewater may be different from other aquatic environments due to different antibiotic application patterns. For example, some antibiotics, such as cefotiam, piperacillin, and vancomycin, are mainly used in hospitals (Kümmerer and Henninger, 2003). Therefore, the aim of this study was to evaluate the pollution levels of antibiotics, ARB, ARGs, and mobile genetic elements (MGEs) in untreated hospital wastewater to identify the potential risks of the discharge of clinical ARB and ARGs into the environment. A broad range of antibiotics from different families, including sulfonamides, tetracyclines, quinolones, macrolides, cephalexin, lincomycin, and trimethoprim, were selected and monitored in the wastewater from three tertiary public hospitals located in Xinxiang city, central China.

2. Materials and methods

2.1. Study sites and sampling

Hospital wastewater samples were collected from three tertiary public hospitals (abbreviated as H1, H2, and H3) located in Xinxiang city, central China, in January and July 2016. The three hospitals are comprehensive hospitals with general departments that each admit 800–1300 patients per day and have bed capacities of 1740, 1200, and 700, respectively. The wastewater was collected from the outflow from daily medical applications in sterile plastic bottles. The collected samples were transported to the laboratory in a portable icebox for immediate processing.

2.2. Quantitation of antibiotics in hospital wastewater with solid-phase extraction and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS)

The procedures for the extraction of the antibiotics were according to Zhang et al. (2011). Briefly, the collected water samples were filtered through 0.45 μm fiber filters. The pH of the filtrate was adjusted to 3.0 with 6 M HCl, and Na_2EDTA was added (to a final concentration of 0.2 g/L) to minimize any interference during the analysis. The extracted antibiotic compounds were analyzed with a Waters UPLC-TQD system (Waters, USA) equipped with a Waters ACQUITY FTN AutoSampler, Waters Xevo TQ MS, and Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm). The specific reaction conditions and method of analysis were according to Gros et al. (2013).

Tetracycline, oxytetracycline, erythromycin, spiramycin, sulfadiazine, sulfamethazine, sulfamethoxazole, cefalexin, ciprofloxacin, ofloxacin, enrofloxacin, norfloxacin, lincomycin, and trimethoprim were purchased from Dr. Ehrenstorfer (Augsburg, Germany) as standard compounds. The standard curve for each antibiotic was constructed with at least five concentrations ($R^2 > 0.99$). The recovery and matrix effects were estimated according to Matuszewski et al. (2003) and are shown in Table 1.

2.3. Enumeration of THCB and CMARB

Colony-forming units (CFUs) of total heterotrophic cultivable heterotrophic bacteria (THCB) and CMARB were measured with a standard plate dilution technique on Luria–Bertani (LB) agar, Salmonellae Shigella agar (SS), or Mueller–Hinton (MH) agar. Samples of 1 mL of hospital wastewater were transferred into centrifuge tube containing 9 mL of stroke-physiological saline solution, and mixed thoroughly. For CMARB culture, tri-resistant media were prepared by mixing three of the eight antibiotics tested, as listed in Table S1. Eight different antibiotics (tetracycline, cephalexin, erythromycin, gentamicin, chloramphenicol, kanamycin, ciprofloxacin, and amoxicillin) were purchased from Solarbio Technology Co., Ltd. (Beijing, China) and used for the culture and isolation of CMARB in hospital wastewater. The working concentrations of these antibiotics were higher than the minimum inhibitory concentrations for resistant bacteria according to CLSI guidelines (Franklin et al., 2012), as listed in Table S2. Plates of LB, SS, or MH agar containing all of the different combinations of antibiotic were inoculated with 0.2 mL of the diluted wastewater sample, and incubated at 25 °C for 48–72 h (Qingxiang et al., 2017). To culture THCB, LB, SS, or MH plates without antibiotics were inoculated and incubated under the same conditions. The abundance of CMARB was determined based on the ratio of CMARB to THCB on each type of agar plate. Each hospital wastewater sample was diluted in a series of consecutive decimal steps and each dilution was plated by triplicate.

2.4. Community characterization of total bacteria (TB) and CMARB

Total DNA was extracted from the three hospital wastewater samples to characterize the communities of TB. Each hospital wastewater sample (200 mL) was filtered through a polycarbonate membrane (0.22 μm; Xinya, Shanghai, China), and the filter membranes were then used for DNA extraction with the E.Z.N.A.® Water DNA Kit (Omega, Solon, OH, USA), according to the manufacturer's instructions. To determine the diversity of CMARB cultivated on the tri-resistant media described above, all colonies were harvested and mixed and the total genomic DNA was extracted. To obtain template DNA from the aggregate colonies present on tri-resistant media, the surface of the agar

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