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Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals[☆]

Edina Szekeres^{a, b}, Andreea Baricz^a, Cecilia Maria Chiriac^{a, b}, Anca Farkas^b, Ocsana Opris^c, Maria-Loredana Soran^c, Adrian-Stefan Andrei^d, Knut Rudi^e, Jose Luis Balcázar^f, Nicolae Dragos^{a, b}, Cristian Coman^{a, *}

^a NIRDBS, Institute of Biological Research, Cluj-Napoca, Romania

^b Molecular Biology and Biotechnology Department, Faculty of Biology and Geology, Babeş-Bolyai University, Cluj-Napoca, Romania

^c National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania

^d Institute for Interdisciplinary Research in Bio-Nano-Sciences, Babeş-Bolyai University, Cluj-Napoca, Romania

^e Department of Chemistry, Biotechnology and Food Sciences, Norwegian University of Life Sciences, Aas, Norway

^f Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, Girona, Spain

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ABSTRACT

Antimicrobial resistance represents a growing and significant public health threat, which requires a global response to develop effective strategies and mitigate the emergence and spread of this phenomenon in clinical and environmental settings. We investigated, therefore, the occurrence and abundance of several antibiotics and antibiotic resistance genes (ARGs), as well as bacterial community composition in wastewater effluents from different hospitals located in the Cluj County, Romania. Antibiotic concentrations ranged between 3.67 and 53.05 $\mu\text{g L}^{-1}$, and the most abundant antibiotic classes were β -lactams, glycopeptides, and trimethoprim. Among the ARGs detected, 14 genes confer resistance to β -lactams, aminoglycosides, chloramphenicol, macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, sulfonamides, and tetracyclines. Genes encoding quaternary ammonium resistance and a transposon-related element were also detected. The *sulI* and *qacEΔ1* genes, which confer resistance to sulfonamides and quaternary ammonium, had the highest relative abundance with values ranging from 5.33×10^{-2} to 1.94×10^{-1} and 1.94×10^{-2} to 4.89×10^{-2} copies/16 rRNA gene copies, respectively. The dominant phyla detected in the hospital wastewater samples were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Among selected hospitals, one of them applied an activated sludge and chlorine disinfection process before releasing the effluent to the municipal collector. This conventional wastewater treatment showed moderate removal efficiency of the studied pollutants, with a 55–81% decrease in antibiotic concentrations, 1–3 order of magnitude lower relative abundance of ARGs, but with a slight increase of some potentially pathogenic bacteria. Given this, hospital wastewaters (raw or treated) may contribute to the spread of these emerging pollutants in the receiving environments. To the best of our knowledge, this study quantified for the first time the abundance of antibiotics and ARGs in wastewater effluents from different Romanian hospitals.

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1. Introduction

Since their introduction in the 1940s, antibiotics have been widely used in human and veterinary medicine to treat infections as well as growth promoters in animal husbandry (Davis and Davies, 2010). The overuse and misuse of these antimicrobial agents has, however, led to an increase and spread of antibiotic-resistant bacteria, and the occurrence of resistant pathogenic

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* Corresponding author. Institute of Biological Research, 48 Republicii street, 400015, Cluj-Napoca, Romania.

E-mail address: cristian.coman@icbcluj.ro (C. Coman).

strains has hampered their clinical use (Rossolini et al., 2014). Although the importance of antibiotic resistance for human health has been recognized decades ago, it is only recently that this phenomenon has been considered as a global health concern with major economic and political implications (Cantas et al., 2013). Consequently, future efforts should be directed to understanding the mechanisms and conditions that contribute to the acquisition and spread of antibiotic resistance, not only in medical but also in environmental settings.

Considering that 20–30% of European patients receive antibiotics during their hospitalization (Ansari et al., 2009), hospital effluents may be considered as hotspots for horizontal gene transfer, which can facilitate inter- and intraspecies transfer of antibiotic resistance determinants and virulence factors (Jackson et al., 2011; Hocquet et al., 2016). Horizontal gene transfer is made possible in large part by the existence of mobile genetic elements (i.e., transposons and integron-associated gene cassettes) that can efficiently contribute to the acquisition, maintenance and spread of antibiotic resistance genes (ARGs) within bacterial communities (Ragan and Beiko, 2009). Class 1 integrons are one of the most successful in the recruitment of these genes among bacterial species from various hospital settings worldwide (Nardelli et al., 2012; Stalder et al., 2014). Hospital effluents also represent a major source of pollutants, such as antibiotics, heavy metals, and disinfectants, which are discharged into sewer systems, and/or receiving surface waters, often without any prior treatment (Cantas et al., 2013; Berendonk et al., 2015). Only a few countries recommend pre-treatment of these wastewaters before their release to water bodies, and no restrictions are foreseen in the European Directive 91/271/EEC for this special category of waste. Moreover, current EU legislation does not include specific regulations, neither the potential presence of antibiotic-resistant bacteria and ARGs in these waters nor their concentration thresholds. The evaluation of ARGs in clinical and environmental settings would therefore be the first step in tackling the rapidly growing resistance to antibiotics. Although the knowledge of antibiotic resistance in hospital wastewaters has largely depended on data provided by culture-based methods and the analysis of a limited number of antibiotics and ARGs (Duong et al., 2008; Li et al., 2013; Fekadu et al., 2015; Hocquet et al., 2016), these data are limited and biased towards cultivable members of the community. Moreover, quantitative data from developing countries are scarce.

A survey of self-medication with antibiotics related that this type of misuse was highest in Eastern Europe, including Romania, where the major groups of antimicrobial drugs used for self-medication were penicillins, macrolide-lincosamide-streptogramin B (MLS_B), tetracyclines, and quinolones (Grigoryan et al., 2006). Although Romania is a leading country in the consumption of certain classes of antibiotics in Europe (Popescu et al., 2016), limited information is available on the consumption of antimicrobials in human healthcare settings (e.g., hospitals, long-term care facilities, and private practices).

Given this, the aim of this study was to determine the occurrence and abundance of antibiotics and ARGs, as well as bacterial community composition in wastewater effluents from different hospitals located in the Cluj County, Romania. We used culture-independent approaches to provide a better understanding of bacterial community composition and function. It should be noted that one of selected hospitals applies an activated sludge and chlorine disinfection process before releasing the effluent to the municipal collector. Raw and treated wastewater samples were thus collected to determine the removal potential of these pollutants.

2. Material and methods

2.1. Sampling procedure

Wastewater effluent samples were collected in June 2015 in triplicate from three hospitals located in different cities from the Cluj County, Romania. Among selected hospitals, hospital 1 (H1) has an oncological profile and a number of 535 beds. Hospital 2 (H2) is a small general hospital with 113 beds, providing service to around 30,000 inhabitants and hospital 3 (H3) has also a general profile with 453 beds for 43,472 inhabitants. H2 and H3 facilities release their raw effluents directly into the wastewater network, whereas the effluent from H1 is treated using an activated sludge and chlorine disinfection process before being discharged into the municipal collector. In this latter case, raw (H1) and treated (H1TWW) wastewater samples were collected for comparative purposes. All wastewater samples were collected using sterile amber-colored containers and transported on ice to the laboratory for analysis.

2.2. Quantification of antibiotics in hospital wastewaters

The preconcentration of antibiotics from the hospital wastewaters was carried out using Oasis HLB SPE cartridges (500 mg, 6 mL; Waters, USA) on a Supelco Visiprep SPE vacuum manifold from Sigma - Aldrich (Germany). Before analysis, the extracts were concentrated using a rotary evaporator (Laborota 4011-digital; Heidolph, Germany) and passed through nylon syringe filters (13 mm × 45 µm; Phenomenex, USA). Before sample application, cartridges were conditioned with 10 mL methanol and 10 mL of ultrapure water. Prior to analysis, 0.2 g EDTA was added to 100 mL sample and the pH was adjusted to 5.5 with 0.5 N HCl or 5% NH₄OH. The samples were passed through the cartridges at a flow rate of 5 mL min⁻¹. The antibiotics retained on the cartridge were eluted with 10 mL methanol, evaporated to dryness using a rotary evaporator at 40 °C and the residues were dissolved in 2 mL of ultrapure water. All extractions were performed in triplicate. The analyses of the antibiotics were carried out using a high-performance liquid chromatography (HPLC) system equipped with diode array and mass spectrometry (MS) detectors (Shimadzu, Japan). The MS was a single quadrupole. The separation of the antibiotics was performed using a Grace Alltima RP-18 column (3 µm, 10 × 0.3 cm; Merck, Germany), at 40 °C. The MS parameters were: capillary voltage of 1.5 kW, 250 °C desolvation temperature and 200 °C interface temperature with positive electrospray ionization (ESI). The concentration of antibiotics in hospital wastewaters was determined by the standard addition method as previously described by Oprea et al. (2016). The recovery rates varied between 97% and 104% (data not shown).

2.3. DNA extraction

Three wastewater samples were collected from each sampling site and were combined to form a representative sample for each hospital (500 mL). These samples were filtered in triplicate through 0.2 µm-pore-size mixed cellulose ester membrane filters (Fioroni, France) (150 mL each) to collect microbial biomass. Each filter was cut into small pieces and used for total DNA extraction using the ZR Soil Microbe DNA kit (ZymoResearch, USA), according to the manufacturer's instructions. The concentration and quality of the extracted DNA were determined with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA samples were stored at -20 °C until further analysis.

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