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Short Communication

Contribution of bacteriophage and plasmid DNA to the mobilization of antibiotic resistance genes in a river receiving treated wastewater discharges

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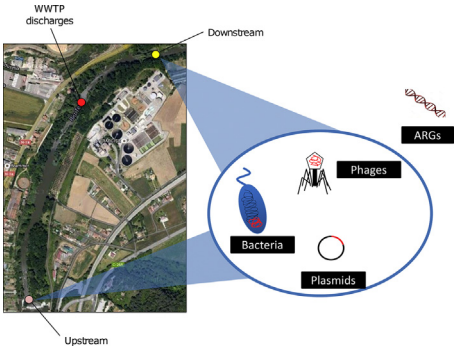
HIGHLIGHTS

- We investigated the presence of antibiotic resistance genes (ARGs) in bacterial, phage and plasmid DNA fractions.
- Higher copy numbers of the target ARGs were detected in the three DNA fractions downstream of WWTP than in upstream sites.
- Genes conferring resistance to β-lactams and glycopeptides only showed significant differences in phage and plasmid DNA.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, we quantified eleven antibiotic compounds and nine antibiotic resistance genes (ARGs) in water samples collected upstream and downstream of the discharge point from a municipal wastewater treatment plant (WWTP) into the Ter River. Antibiotics were analyzed by liquid chromatography coupled to mass spectrometry, whereas the concentration of ARGs in bacterial, phage and plasmid DNA fractions was determined by real-time PCR to explore their contribution to environmental antibiotic resistance. WWTP discharges resulted in higher concentrations of antibiotic residues as well as ARGs in water samples collected downstream the impact point. Specifically, genes conferring resistance to macrolides (*ermB*), fluoroquinolones (*qnrS*) and tetracyclines (*tetW*) showed significant differences ($p < 0.05$) between upstream and downstream sites in the three DNA fractions (*i.e.* bacteria, plasmids and phages). Interestingly, genes conferring resistance to β-lactams (bla_{TEM}, bla_{NDM} and bla_{KPC}) and glycopeptides (*van*A) only showed significant differences ($p < 0.05$) between upstream and downstream sites in phage and plasmid DNA but not in the bacterial DNA fraction. Our results show for the first time the extent to which phages and plasmids contribute to the mobilization of ARGs in an aquatic environment exposed to chronic antibiotic pollution *via* WWTP discharges. Accordingly, these mobile genetic elements should be included in further studies to get a global view of the spread of antibiotic resistance.

1. Introduction

Although antibiotics have saved millions of lives since their discovery, their extensive use in human medicine, animal husbandry and

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agriculture has resulted in the emergence and spread of antibiotic-resistant bacteria. Resistance to antibiotics may result from innate (intrinsic) or acquired mechanisms. Intrinsic resistance is a naturally occurring phenomenon that may be characteristic for a given strain or species, whereas acquired resistance can be due to either genetic mutations or acquisition of foreign genetic material from other bacteria (Blair et al., 2015). This latter process, referred to as horizontal gene transfer, is mainly driven by mobile genetic elements (MGEs) such as plasmids, insertion sequences, insertion sequence common region (IS*CR*) elements, transposons, genomic islands, integrating conjugative elements and bacteriophages, which largely contribute to evolution and diversity of bacterial genomes through the acquisition and recombination of foreign DNA (Frost et al., 2005). This set of MGEs, also known as the "mobilome", play a crucial role in the recruitment, maintenance and spread of genes conferring resistance to antimicrobial agents and heavy metals, and genes encoding virulence factors in bacterial pathogens (Gillings, 2013). The role of these MGEs in the acquisition and spread of antibiotic resistance genes (ARGs) has been extensively studied, particularly in clinical isolates (McCarthy et al., 2014; Mikalsen et al., 2015). However, few studies have addressed their contribution to antibiotic resistance in environmental settings (Parsley et al., 2010; Kristiansson et al., 2011; Perry and Wright, 2013; Lekunberri et al., 2017).

Previous studies suggested that municipal wastewater treatment plants (WWTP) are potential hotspots for the acquisition and exchange of ARGs among resident bacteria (Rizzo et al., 2013; Pruden, 2014), which are subsequently released into the environment through their effluents. Although the concentration of ARGs is usually higher at river sites located downstream the discharge point (Amos et al., 2014; Berglund et al., 2015; Proia et al., 2016), the contribution of MGEs to the prevalence of environmental antibiotic resistance has not extensively been explored. The present study is then aimed to determine the impact of a WWTP effluent discharge on both the antibiotic pollution and abundance of eleven ARGs in plasmid and phage DNA fractions to assess the contribution of these MGEs in the spread of resistance in the receiving river. Additionally, the abundance of ARGs in bacterial DNA fractions was also analyzed for comparative purposes.

2. Materials and methods

2.1. Study area and sampling sites

Water samples were collected on April 5th, 2016, from the Ter River upstream (900 m) and downstream (280 m) of the Campdorà WWTP effluent discharge (42 01.597 N, 02 49.809 E). This WWTP, which uses a conventional activated sludge process, receives untreated hospital wastewater (1000–1500 $m^3 \cdot day^{-1}$) together with municipal sewage from the city of Girona, Catalunya (45,000–55,000 $m^3 \cdot day^{-1}$). Moreover, the Ter River is a primary source of drinking water for the population of the Girona region (738,682 inhabitants in 2015) as well as for the Barcelona metropolitan area (4,776,107 inhabitants in 2015). At each sampling point, six water replicates (1000 ml each) were collected at 1-min intervals and stored at 4 °C in a portable icebox until arrival at the laboratory.

2.2. Quantification of antibiotics

For the determination of antibiotic compounds, the analysis was performed following the method described in Gros et al. (2013). Briefly, water samples were filtered through 0.45-mm-pore-size membrane filters (PVDF), followed by sample acidification at pH 2.5 with HCl. After pH adjustment, an appropriate amount of 0.1 M NA_2EDTA solution was added. A volume of 100 ml of river water was preconcentrated using OASIS HLB cartridges (60 mg, 3 ml) (Waters Corp.; Mildford MA, USA). Instrumental analysis was done by ultra-high performance liquid chromatography (Waters Corp.) coupled to a quadrupole linear-ion trap mass spectrometer (AB Sciex, Foster City, CA, USA). The chromatographic separation was achieved using an Acquity HSST3 (50 mm × 2.1 mm i.d., 1.8 mm particle size) also from Waters. The mobile phase was acetonitrile and acidified HPLC grade water (0.1% formic acid). Analytes were identified under the positive electrospray ionization and the mass spectrometer was operated in the multiple reaction-monitoring mode (MRM), selecting two transitions per compound (the first transition used for quantification and the second one for confirmation). For the quantification we used the internal standard method using an isotopically labelled compound for each chemical group of antibiotics.

2.3. DNA extraction

Water samples (1000 ml each) were filtered through 0.22-μm-pore-size polycarbonate filters, which allowed retention of bacterial cells. Half of the biomass, recovered from 500 ml of water, was used to extract bacterial DNA, while the other half was used to extract plasmid DNA. For bacterial DNA extraction, the collected biomass was resuspended in standard lysis buffer and digested with lysozyme (20 $mg \cdot ml^{-1}$) and proteinase K (10 $mg \cdot ml^{-1}$). Genomic DNA was then extracted using a standard phenol-chloroform method. For plasmid DNA extraction, the collected biomass was resuspended in alkaline lysis buffer (0.2 N NaOH; 1.0% SDS), followed by potassium acetate (3.0 M, pH 5.5) precipitation of chromosomal DNA, and plasmid DNA was then extracted using a standard phenol–chloroform method and ethanol precipitation. For phage DNA extraction, phage particles from the filtrates were precipitated using polyethylene glycol (PEG 6000), centrifuged at 14,000*g* for 10 min, and the obtained pellet was treated with DNase (100 $U \cdot ml^{-1}$) to remove free DNA. The extraction of phage DNA was performed as described previously by Colomer-Lluch et al. (2011). The DNA concentration was measured using Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA) and purity was determined by measuring A260/A230 and A260/A280 absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA).

2.4. Quantification of ARGs

The copy number of selected genes conferring resistance to the main antibiotics used for treating bacterial infections in humans and animals, such as β-lactams (bla_{KPC}, bla_{NDM} and bla_{TEM}), fluoroquinolones (*qnrS*), tetracyclines (*tet*O and *tet*W), sulfonamides (*sul*I), glycopeptide antibiotics (*van*A) and macrolides (*ermB*) was determined by real-time PCR (qPCR). All qPCR assays were conducted on an Mx3005P system (Agilent Technologies), according to previously described conditions (Marti et al., 2013; Subirats et al., 2017). Briefly, an initial denaturation at 95 °C for 3 min was applied, followed by 40 cycles at 95 °C for 15 s and at the annealing temperature given in Supplementary Table S1 for 20 s. All reactions were performed in triplicate and contained a total volume of 30 μl, including 1 μl of template, primer concentrations between 0.2 and 0.6 μM, and 2× Brilliant III Ultra Fast QPCR Master Mix (Agilent Technologies; Santa Clara, CA, USA). Specific primer sets were used for amplify each gene (Supplementary Table S1).

2.5. Statistical analysis

After being tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test), mean values of six replicates for each sample were compared using the Student's *t*-test to determine if there was significant variation between upstream and downstream water samples. Statistical analysis was conducted using SPSS version 17.0 (SPSS; Chicago, IL, USA) and statistical significance was determined at $p < 0.05$.