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The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment☆

William Calero-Cáceres, Javier Méndez, Julia Martín-Díaz, Maite Muniesa*

Department of Genetics, Microbiology and Statistics, University of Barcelona, Diagonal 643, Annex, Floor 0, E-08028 Barcelona, Spain

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ABSTRACT

The spread of antibiotic resistance genes (ARGs) in the environment is a serious concern. Bacterial ARGs can spread via different mobile genetic elements as phage particles, which thereby emerge as novel vectors for environmental dissemination. To assess how climate events, such as heavy rains or water scarcity, could affect the spread of ARGs, it is necessary to know their prevalence and abundance in aquatic environments as well as the potential reservoirs from which they could become mobile. This study evaluates the occurrence of ARGs in the water and sediment of a Mediterranean river. Six clinically relevant ARGs (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *qnrA*, *qnrS*, *mecA* and *sul1*) were quantified by qPCR in the bacterial and phage fractions of 69 water and 70 sediment samples from the River Llobregat (NE Spain), collected during both dry and rainy periods. *bla*$_{TEM}$ and *sul1* were the most prevalent and abundant ARGs; the others were more variable. Significant seasonal differences in ARG prevalences and abundances were observed. Since ARGs were detected in the sediment, the persistence of the most abundant ARGs naturally occurring in that sediment (*bla*$_{TEM}$ and *sul1*) was evaluated under three conditions. No ARG inactivation occurred in fresh sediment over 14 days; while the ARGs declined by less than 2 $\log_{10}$ units over 35 days in semi-dry and dry sediment. The occurrence of ARGs in water and sediment is influenced by seasonal conditions and they can be mobilized by bacteria and phage particles. In sediment, ARGs persist for long periods and hence sediment can be a natural reservoir of ARGs, from where they can spread and cause the emergence of new resistant strains.

1. Introduction

Since their introduction, the misuse and overuse of antibiotics has played a crucial role in the development of antibiotic resistances (Hall, 2004; Roca et al., 2015; Rodríguez-Rojas et al., 2013). The emergence of multiresistant bacteria poses a threat to the treatment of bacterial infections. Antibiotic resistance can be generated by mutation or acquired via antibiotic resistance genes (ARGs) which can become mobile through horizontal gene transfer (HGT) across bacterial populations. The most commonly studied mobile genetic elements that permit dissemination of ARGs are plasmids, transposons and phages (Muniesa et al., 2013).

Given that many bacterial and fungal genera found in diverse environments carry resistance determinants (Davies and Davies, 2010), it is conceivable that many ARGs originate from the environment and persist in environmental reservoirs. This phenomenon may provide a source of ARGs for organisms that produce antibiotics as well as for those that do not (Cantón, 2009; Baquero et al., 2008). Studies conducted in freshwater reservoirs and surface water (Czekalski et al., 2012; Marti et al., 2014; Stoll et al., 2012), human sewage (Michael et al., 2013; Munir et al., 2011), animal faecal waste (Colomer-Lluch et al., 2011b; Economou and Gousia, 2015; Hsu et al., 2014), reclaimed water (Fahrenfeld et al., 2013; Huang et al., 2011), sewage sludge (Auerbach et al., 2007; Calero-Cáceres et al., 2014), river sediment (Berglund et al., 2014; Luo et al., 2010; Pei et al., 2006) and soil (Nesme and Simonet, 2015) support the hypothesis of the environmental origin of ARGs.

Many studies have focused on ARG codification in plasmids or transposons, but recent studies highlight the contribution of phages to the dissemination of ARGs (Colomer-Lluch et al., 2011b). Phages might be particularly relevant in the environment, where phage capsids could better protect the DNA from degradation and where no direct cell-to-cell contact is required (Colomer-Lluch et al., 2011b). The factors contributing to the spread of ARGs via

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* Corresponding author.
E-mail address: mmuniesa@ub.edu (M. Muniesa).

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any vehicle from their environmental reservoirs to clinical settings should be further explored.

Climatic variations are considered to be factors that potentially influence ARG dissemination (Laxminarayan et al., 2013). The Mediterranean region, with its seasonal fluctuations, is highly vulnerable to such variations. The Mediterranean basins constitute one of the most water-stressed areas in the world, with regard to the possible consequences of climate change. Records indicate a decrease in temporal trends and stable long-term precipitation patterns over the last few decades (Agència Catalana de l'Aigua, 2009; Turco and Llasat, 2011). Despite this decrease, the region will continue to be affected by both heavy rain events and droughts (IPCC, 2008).

The River Llobregat is a Mediterranean river that is used in this study as a model. It suffers a moderate impact mostly from humans and industrial effluents (Sabater et al., 2015), diffuse pollution from animal origin (either farms or wild animals), and as previously reported, it contains a significant amount of antibiotic drug products (Cabeza et al., 2012; Osorio et al., 2012; Sabater et al., 2012).

To quantify potential health risks, it is important to identify different environmental reservoirs of ARGs, how ARGs can persist there and how climate events can affect the mobilization of ARGs from these reservoirs. The aim of this study was to evaluate the occurrence of six clinically relevant ARGs in the bacteria and bacteriophage fractions of water and sediment from the River Llobregat. To confirm the role of the sediment as a reservoir of ARGs, the persistence of ARGs in the river sediment was measured using *in vitro* mesocosms that simulate natural conditions.

2. Material and methods

2.1. Samples

69 water and 70 sediment samples were collected between September 2012 and December 2014 in the lower course of the River Llobregat (Catalonia, NE Spain) (Fig. 1). The mean annual precipitation over these years in the river catchment area is 3330 hm^3, and the average annual discharge is 693 hm^3 (Agència catalana de l'aigua, 2016; Köck-Schulmeyer et al., 2011; Muñoz et al., 2009). The samples were collected monthly during dry periods and via sampling campaigns organized 24–48 h after rainfall events. On the sampling date, information on water temperature, streamflow, solar irradiance and accumulated precipitation was obtained. The temperatures and irradiation rates on those days were obtained from the database of the regional weather service (*Servei Metereològic de Catalunya*).

All samples were collected in sterile containers, transported to the laboratory at 5 °C ± 2 °C within two hours of collection, and processed immediately for microbiological analysis and further experiments.

2.2. Evaluation of E. coli and somatic coliphages

To provide faecal pollution levels of the water and sediment samples, two indicators of faecal pollution, *E. coli* and somatic coliphages, were quantified. *E. coli* was enumerated following standardized methods (ISO 9308) (Anonymous, 2014) with few modifications. Briefly, serial decimal dilutions of the water samples and decimal dilutions of a homogenate of the sediment using PBS at pH 7.4 (1:10 *w:v*) in a vertical shaker for 30 min at 900 osc/min, were filtered through membrane filters with a 0.45 μm pore diameter (47 mm, white gridded, EZ-Pak® Membrane Filters, Millipore, Bedford, MA). The membranes were placed upside up on Chromocult® Coliform Agar for *E. coli* quantification, and incubated at 36 °C for 18 h. Based on our previous observations, blue-violet colonies were always *E. coli* and as such were considered without further confirmation using oxidase test. Somatic coliphages were quantified following ISO 10705-2 (Anonymous, 2000). To this end, the river water samples were filtered through low protein-binding membrane filters with a 0.22 μm pore size (Millex-GP, Millipore, Bedford, MA), decimally diluted and processed following the standard procedure using *E. coli* WG5 (ATCC 700078) as the host. All samples were analysed in triplicate.

2.3. Bacterial DNA extraction

To extract bacterial DNA from the river water, 100 mL of the water samples was filtered through membrane filters with a 0.45 μm pore diameter (47 mm, white gridded, EZ-Pak® Membrane Filters, Millipore). The membranes were rinsed twice with 10 mL phosphate-buffered saline (PBS) solution which was filtered through them to reduce the number of viral particles present in the samples and retained in the membranes (Muniesa et al., 2005). The bacterial content of the membranes was recovered by washing the membranes in 10 mL of Luria Bertani broth (LB). The suspensions were centrifuged at 3000×*g* for 10 min and the pellet was suspended in 200 μL of LB broth. Bacterial DNA was extracted using Macherey-Nagel NucleoSpin® Blood (Düren, Germany) following the manufacturer's instructions. For bacterial DNA from sediment, the samples were homogenized using PBS at pH 7.4 (1:10 *w:v*) in a vertical shaker for 30 min at 900 osc/min. The suspension was then centrifuged at 3000×*g* for 10 min. The bacterial DNA was extracted from the pellet using the PowerSoil® DNA extraction kit from Mobio Laboratories Inc (Carlsbad, CA) and following the manufacturer's instructions.

2.4. Bacteriophage DNA extraction

To purify the bacteriophage fraction of the water samples, 100 mL of each sample was centrifuged at 3000×*g* for 10 min and the supernatant was filtered, concentrated, extracted and purified following the method described below.

Phages from sediment samples were extracted using a previously validated protocol for a similar matrix (Calero-Cáceres et al., 2014). Briefly, the sediment was homogenized using PBS at pH 7.4 (1:10 *w:v*) in a vertical shaker for 30 min at 900 osc/min. The suspension was then centrifuged at 3000×*g* for 5 min and the supernatant was filtered through low protein-binding membrane filters with a 0.22 μm pore size (Millex-GP, Millipore). Viral particles were 100-fold concentrated by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore) to a final volume of 1 mL. The phage suspension was treated with chloroform 1:10 (*v:v*) to eliminate possible membrane vesicles containing DNA that might be present in the samples. The chloroform-treated samples were vortexed and centrifuged at 16,000×*g* for 10 min, and the aqueous phase was recovered. The supernatant was treated with DNase (100 units/mL of the phage lysate at 37 °C for 1 h), to remove non-packaged DNA. The DNase was heat inactivated at 80 °C for 10 min. Phage DNA was extracted using proteinase K digestion and phenol-chloroform (1:1, v:v) (Calero-Cáceres et al., 2014). The concentration and purity of the phage DNA extracted was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). To verify the absence of non-packaged DNA, the protocol for DNA extraction from the phage fraction of the samples was always accompanied by several controls as described previously (Colomer-Lluch et al., 2014a).

2.5. qPCR procedures

This study focused on six clinically relevant ARGs that had

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