Water Research 95 (2016) 11–18

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater

William Calero-Cáceres, Maite Muniesa*

Department of Microbiology, University of Barcelona, Diagonal 643. Annex. Floor 0, E-08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
22 February 2016
Accepted 1 March 2016
Available online 3 March 2016

Keywords:
Antibiotic resistance
Bacteriophages
Wastewater
Public health

ABSTRACT

The emergence and prevalence of antibiotic resistance genes (ARGs) in the environment is a serious global health concern. ARGs from bacteria can be mobilized by mobile genetic elements, and recent studies indicate that phages and phage-derived particles, among others, could play a role in the spread of ARGs through the environment. ARGs are abundant in the bacterial and bacteriophage fractions of water bodies and for successful transfer of the ARGs, their persistence in these environments is crucial. In this study, three ARGs (bla_{TEM}, bla_{CTX-M} and *sul1*) that naturally occur in the bacterial and phage fractions of raw wastewater were used to evaluate the persistence of ARGs at different temperatures (4 °C, 22 °C and 37 °C) and pH values (3, 7 and 9), as well as after various disinfection treatments (thermal treatment, chlorination and UV) and natural inactivation in a mesocosm. Gene copies (GC) were quantified by qPCR; then the logarithmic reduction and significance of the differences between their numbers were evaluated. The ARGs persisted for a long time with minimal reductions after all the treatments. In general, they showed greater persistence in the bacteriophage fraction than in the bacterial fraction. Comparisons showed that the ARGs persisted under conditions that reduced culturable *Escherichia coli* and infectious coliphages below the limit of detection. The prevalence of ARGs, particularly in the bacteriophage fraction, poses the threat of the spread of ARGs and their incorporation into a new bacterial background that could lead to the emergence of new resistant clones.

1. Introduction

The development of antibiotic resistance is a common evolutionary process in microorganisms to ensure their survival against other microorganisms. Antibiotic resistance can appear through a process of spontaneous mutation or be acquired via vertical or, more commonly, horizontal gene transfer. The use and misuse of antibiotics over recent decades and the presence of certain concentrations of antibiotics in different environments could have accelerated this phenomenon by exerting a selective pressure. Poor sanitary conditions, inefficient (or the total absence of) sewage treatment, and deficiencies in the control of both human and veterinary infections stimulate the generation and further spread of antibiotic resistant determinants (EFSA-ECDC, 2015; World Health Organization, 2014). The unfortunate consequences are that an estimated 25,000 people in Europe (ECDC/EMEA, 2009) and 23,000 in America die every year because of antibiotic-resistant infections (The White House, 2014).

Antibiotic resistance genes (ARGs) are abundant in water from different bodies of water with different levels of faecal pollution (Li et al., 2015; Rodriguez-Mozaz et al., 2014; Xu et al., 2014). One challenge we face is to evaluate whether the ARGs retain biological activity and therefore remain available to horizontal gene transfer mechanisms, which could lead to the emergence of new resistant clones. Despite their occurrence, detection of ARGs is not included in the analysis of the risk posed by water from different sources. In accordance with European regulations, water recovered from sewage only has to comply with different levels of quality concerning the presence of *Escherichia coli*, *Legionella*, *Taenia* and nematodes as biological parameters (BOE 1620, 2007).

Wastewater treatment processes normally result in significant reductions in the concentration of microorganisms present in sewage, prior to discharge (Lucena et al., 2004; Marín et al., 2015). However, a considerable amount of resistant bacteria are still found in treated sewage (Guardabassi et al., 2002; Huang et al., 2012; LaPara et al., 2011). In addition, under certain inactivating environmental conditions or disinfection treatment, the overall

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

http://dx.doi.org/10.1016/j.watres.2016.03.006
0043-1354/

concentration of bacteria decreases, but the percentage of antibiotic resistant bacteria and consequently of ARGs in the total bacterial community could increase during wastewater treatment (Czekalski et al., 2012; Zhang et al., 2009).

Previous studies have evaluated the persistence of ARGs in bacteria following different disinfection processes: chlorination, UV irradiation and ozonation. According to Auerbach et al., 2007, UV irradiation does not affect the number of detectable tet^R gene types. In contrast, other authors using different chlorine concentrations (Yuan et al., 2015) or high UV doses up to 4000 or 124,770 J/m^2 (Zhuang et al., 2014; McKinney and Pruden, 2012), demonstrate a significant effect on the level of activity of ARGs in wastewater. However, despite ARGs being reduced significantly, a large number were still present after treatment.

Recent studies highlight the role of bacteriophage particles as ARG vehicles in the environment, in accordance with their abundance in human and animal wastewater, surface water and sludge (Calero-Cáceres et al., 2014; Colomer-Lluch et al., 2011, 2014b, 2014a; Marti et al., 2014, 2013). Phage particles containing ARGs enter these biomes from autochthonous bacteria or from the fecal source or pollution (Quirós et al., 2014). There is no information about how disinfection treatments and inactivation processes affect the ARGs present in the bacteriophage fraction, but bacteriophages are known to persist more than bacteria after disinfection procedures (Allué-Guardia et al., 2014; Cantalupo et al., 2011; Wommack et al., 1996). Here, we evaluate the presence of ARGs in the bacteriophage fraction and compare it with their presence in the bacterial fraction when affected by different environmental conditions, and also after different disinfection and natural inactivation processes.

2. Materials and methods

2.1. Samples

Raw urban wastewater samples were collected between autumn 2014 and summer 2015 from the influent of a waste water treatment plant that serves approximately 500,000 people in the Barcelona metropolitan area. All the samples were collected in sterile containers, transported to the laboratory at 5 °C ± 2 °C within 2 h of collection and processed immediately for bacterial counts and further experiments. The samples were used as received for UV irradiation, temperature and pH stability, and natural inactivation; and were 1/10 diluted in deionized water for chlorination.

2.2. Bacterial strains and media

For the detection of *E. coli*, Chromocult® Coliform Agar (Merck, Darmstadt, Germany) was used (International Organization for Standardization, 2014). *E. coli* WG5 (ATCC 700078) was used as a host for the evaluation of somatic coliphages. Quality control procedures shadowed each experiment using phage ΦX174 (ATCC 13706-B1) as the reference material, previously prepared in accordance with the corresponding ISO standard 10705-2 (International Organization for Standardization, 2000). Luria-Bertani (LB) agar or broth was used for routine bacterial propagation to prepare the standards for qPCR assays.

2.3. Stability at different temperatures and pH values

Aliquots of wastewater were placed in sterile tubes at 4 °C, 22 °C, and 37 °C. For the pH assays, the medium was adjusted to pH 3, 7, and 9, using hydrochloric acid, 1 N, and sodium hydroxide, 1 N, and placed in sterile tubes at 4 °C. The samples were removed at days 0, 1, 3, 7, 14, 21, 28 and 42, and the pH was verified after each incubation time.

2.4. Inactivation experiments

Aliquots of wastewater or dilutions thereof were used for the inactivation experiments. For chlorination, we diluted the samples using deionized water, to 1/10. Then we treated them with 10 ppm chlorine supplied as sodium hypochlorite. Hypochlorite solution was calculated to provide the indicated amounts of total residual chlorine in the mixture as previously reported (Durán et al., 2003). The aliquots (50 mL) were removed after 1, 3, 5, 10 and 30 min. Residual chlorine was neutralized by the addition of sodium thiosulphate. For thermal treatment, the tubes were placed into a water bath at 60 °C and 80 °C and were removed after 30 and 60 min. For the UV inactivation, an 8-W, germicidal UV lamp (model G30T8; 0.099-mW/cm2 irradiance at a 253.7-nm wavelength; Sankyo Denki, Tokyo, Japan) was used. The lamp was warmed up for at least 15 min before starting the experiments. The UV dose was calculated using the equation $D = I \times T$, where D is the dose, T is the exposure time and I is the "fluence rate" (or intensity) of the lamp. The samples (10 mL) were statically placed in open petri dishes of 90 mm diameter at 10 cm from the lamp and were removed after 1, 5, 10 and 30 min that corresponded to estimated UV doses of 5.94, 29.7, 59.4, and 178.2 mJ/cm2 at each time. The approach allowed comparison between microorganisms, as shown previously (Allué-Guardia et al., 2014).

To evaluate natural inactivation in a mesocosm, dialysis tubes (cutoff: 14 kDa) were filled with 1/2 diluted wastewater (50 mL). The tubes were sealed and placed in an outdoor artificial pond (60 m^3; non-chlorinated water) protected by a cage, at a depth of 10 cm and were removed after 1, 3, 7, 14, 21 and 28 days (Fig. 3A). The experiments were carried out in winter 2015 (December-February) and summer 2015 (June-August).

Temperatures and irradiation rates were obtained from the regional weather forecast service database (*Servei Metereològic de Catalunya*, Spain).

2.5. Microbiological parameters

For *E. coli* enumeration, serial decimal dilutions of the wastewater samples were filtered through 0.45 μm-pore-diameter membrane filters (47 mm, white gridded, EZ-Pak® Membrane Filters, Millipore, Bedford, MA). The membranes were placed upside up on Chromocult® Coliform Agar for *E. coli* and incubated at 44 °C for 18 h, following the standard procedure ISO 9308-1:2014 (International Organization for Standardization, 2014). To evaluate somatic coliphages, wastewater samples were filtered through low-protein-binding 0.22 μm-pore-size membrane filters (Millex-GP, Millipore, Bedford, MA), the filtrates were decimal diluted and assayed using the double agar method following the standard procedure ISO 10705-2 (International Organization for Standardization, 2000). All the samples were analysed in triplicate.

2.6. DNA extraction

To extract bacterial DNA from the wastewater samples, 25 mL of each sample was filtered through 0.45 μm pore-diameter membrane filters (47 mm, white gridded, EZ-Pak® Membrane Filters; Millipore). The membranes were rinsed twice in 10 mL PBS filtered through the membranes to reduce viral particles (Muniesa et al., 2005). The bacterial content of the membranes was suspended in LB broth. The suspensions were centrifuged at a 3,000 *g* for 10 min and the pellet was suspended in 200 μL of LB. Bacterial DNA was extracted using Macherey-Nagel NucleoSpin® Blood (Düren, Germany), following the manufacturer's instructions.