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The influence of the autochthonous wastewater microbiota and gene host on the fate of invasive antibiotic resistance genes

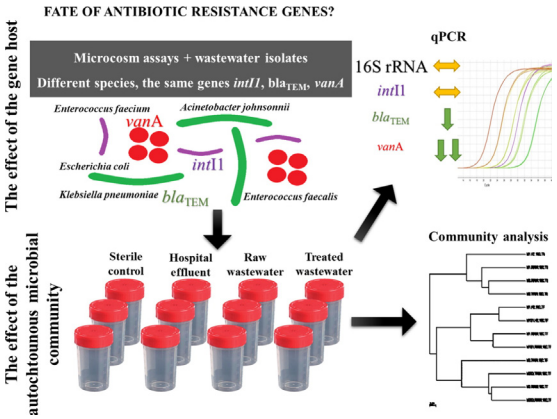
Carlos Narciso-da-Rocha, Célia M. Manaia *

Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

HIGHLIGHTS

- The fate of invasive antibiotic resistance in wastewater was studied.
- *bla*$_{TEM}$ and *van*A genes present lower persistence in water than the 16S rRNA or *int*I1.
- The host of the gene *bla*$_{TEM}$, but not of *van*A, influenced its persistence in water.
- The autochthonous wastewater microbiota may outcompete invasive bacteria.
- The decrease *Gammaproteobacteria* was related with the decrease of resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to assess the fate of invasive antibiotic resistance genes (ARG) discharged in wastewater. With this objective, antibiotic resistant bacteria (ARB) known to harbor specific ARG were inoculated in wastewater (hospital effluent, or municipal raw and treated wastewater) and in ultra-pure sterile water microcosms. Two sets of wastewater ARB isolates were used - set 1, *Enterococcus faecalis*, *Acinetobacter johnsonii*, *Klebsiella pneumoniae* and set 2, *Enterococcus faecium*, *Acinetobacter johnsonii*, *Escherichia coli*. Non-inoculated controls were run in parallel. Samples were collected at the beginning and at the end (15 days) of the incubation period and the abundance of the genes 16S rRNA, *int*I1, *bla*$_{TEM}$ and *van*A and the bacterial community composition were analyzed.
In general, the genes *bla*$_{TEM}$ and *van*A had lower persistence in wastewater and in ultra-pure water than the genes 16S rRNA or the class 1 integron integrase *int*I1. This effect was more pronounced in wastewater than in ultra-pure water, evidencing the importance of the autochthonous microbiota on the elimination of invasive ARG. Wastewater autochthonous bacterial groups most correlated with variations of the genes *int*I1, *bla*$_{TEM}$ and *van*A were members of the classes *Gammaproteobacteria*, *Bacilli* or *Bacteroidia*. For *bla*$_{TEM}$, but not for *van*A, the species of the ARB host was important to determine its fate. These are novel findings on the ecology of ARB in wastewater environments.

1. Introduction

The discharge of antibiotic resistant bacteria (ARB) of human and animal origin in natural land and waterways is considered a serious

* Corresponding author.
E-mail address: cmanaia@porto.ucp.pt (C.M. Manaia).

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environmental problem (Pruden, 2014; Vaz-Moreira et al., 2014; Berendonk et al., 2015). In part, this is due to the fact that some anthropogenic sources such as domestic effluents, even after treatment, are responsible for loading in the environment appreciable amounts of ARB and their genes (ARG). In comparative reviews of different domestic wastewater treatment plants, it was observed that irrespective of the geographic region or treatment process used, these facilities discharged, per minute, ~10^9 ciprofloxacin resistant coliforms and >10^{15} copies of ARG encoding resistance to beta-lactams, tetracycline, sulfamethoxazole or fluoroquinolones (Manaia et al., 2016; Vaz-Moreira et al., 2014). These ARB&ARG have origin in human and animal sources and once they are discharged in the wastewater treatment plants, some will persist being released into the environment (Rizzo et al., 2013; Manaia et al., 2016). These sources of contamination are probably related with the observed accumulation and persistence of ARB&ARG in the environment that is particularly evident for old generation antibiotics such as sulfamethoxazole or amoxicillin, for which resistance prevalence can reach values above 40% in municipal wastewater (Novo et al., 2013; Rizzo et al., 2013; Varela et al., 2014). In spite of these evidences, not much is known about the fate of ARB&ARG that are discharged in complex microbial communities. The observation of genetically closely related ARB in different environments, such as wastewater and some wild life species, suggest that some bacterial lineages with higher environmental fitness may have a major role in the dissemination of ARB&ARG (Leclercq et al., 2013; Vredenburg et al., 2014; Varela et al., 2015). In this aspect it can be hypothesized that both the ARB fitness and the competitive effect of the autochthonous microbiota will be determinant on the fate of a given ARG. Considering the complexity of this issue, this study aimed at establishing a simple model to assess the importance of the ARB species hosting an ARG and of the autochthonous microbiota on the fate of a given ARG. The assays were performed in microcosm settings, composed of ultra-pure sterile water or wastewater samples of different origins, inoculated or not with ARB harboring known ARG, and incubated for two weeks, at 18 °C or 30 °C. Variations of the wastewater bacterial community composition and on the load of the selected ARG were assessed in parallel and possible correlations between both, bacterial community and ARGs, were determined.

2. Material and methods

2.1. ARB&ARG

Five multi-drug resistant (MDR) bacteria exhibiting resistance to antibiotics belonging to at least four different classes and harboring known ARG were used as surrogates in this study. Surrogate bacteria, isolated from wastewater in previous studies (Varela et al., 2013; Varela and Manaia, 2013), were used in two sets of three, as follows: surrogate set 1 (S1) was composed of *Enterococcus faecalis* strain H1EV10 (hospital effluent, *van*A positive), *Acinetobacter johnsonii* strain H1PC5 (hospital effluent, *int*I1 positive) and *Klebsiella pneumoniae* strain H1FC25 (hospital effluent, bla_{TEM} and *int*I1 positive), and surrogate set 2 (S2) comprised *Enterococcus faecium* strain E4EC3 (treated municipal wastewater, *van*A positive), *Acinetobacter johnsonii* strain H1PC5 (hospital effluent, *int*I1 positive) and *Escherichia coli* strain A5EL5 (raw municipal wastewater, bla_{TEM} and *int*I1 positive) (Table 1). Luria Broth (Oxoid, Basingstoke, England) axenic cultures, incubated overnight at 37 °C, were mixed in equal proportions to prepare surrogate mixtures S1 or S2, at a final density of 3.0×10^7 colony forming units (CFU/mL), and were used to inoculate the microcosms.

2.2. Microcosm assays

Two sets of microcosms (M1 and M2) were prepared, each comprising assays in ultra-pure sterile water and in different types of wastewater, inoculated with the surrogate mixed cultures S1 or S2 described above. Non-inoculated microcosms manipulated under the same conditions except addition of surrogate were used as controls. In microcosms M1, wastewater was from hospital effluent (M1-HE) or from raw wastewater of the treatment plant that receives the hospital effluent (M1-RWW). In microcosms M2, wastewater was from the raw (M2-RWW) or treated wastewater (M2-TWW) of a treatment plant that does not receive hospital effluents (Table 1). Inoculated microcosms, named as M1S1 and M2S2, contained 1.0×10^5 CFU/mL of each species used to prepare the surrogate mixture. Surrogate S1 was used in M1 (M1S1) and of surrogate S2 in M2 (M2S2) (Table 1). Microcosms prepared with ultrapure sterile water were used as controls for the absence of autochthonous microbiota, and were designated S1-UP and S2-UP, respectively. All microcosm assays were prepared in 200 mL disposable

Table 1
Experimental setup: microcosms using hospital effluent (HE), raw wastewater (RWW), and treated wastewater (TWW), before incubation (T0) and after incubation (TF), non-inoculated (M1 and M2) or inoculated (M1S1 and M2S2) with surrogate bacteria isolated from wastewater and harboring known antibiotic resistance genes.

Water characteristics										
Microcosm		Quality indicators (average values)			Antibiotics and metals (μg/L)*					
		CQO (mg O_2/L)	BOD (mg O_2/L)	TSS (mg/L)	Tet	Pen	Sul	Cip	As	Hg
M1	HE[a]	622	278	305	2.1	1.4	1.5	2.5	6.5	1.2
	RWW[a]	699	488	334	4.2	2.2	3.0	0.9	4.9	0.3
M2	RWW[b]	400	250	N.A.	18.0	12.0	4.5	5.0	2.8	—
	TWW[b]	91	23	N.A.	5.8	19.0	1.4	1.9	2.1	—

Surrogates				
Microcosm		*van*A	*int*I1	bla_{TEM}
M1S1	HE RWW UP	*Enterococcus faecalis* (H1EV10)[c]	*Acinetobacter johnsonii* (H1PC5)[a] *Klebsiella pneumoniae* (H1FC25)[a]	*Klebsiella pneumoniae* (H1FC25)[a]
M2S2	RWW TWW UP	*Enterococcus faecium* (E4EC3)[c]	*Acinetobacter johnsonii* (H1PC5)[a] *Escherichia coli* (A5EL5)[b]	*Escherichia coli* (A5EL5)[b]

[a] Varela et al. (2015).
[b] Ferreira da Silva et al. (2007).
[c] Varela et al. (2013).
* Maximum values of tetracycline (Tet), penicillin G (Pen), sulfamethoxazole (Sul), ciprofloxacin (Cip) and arsenic (As) detected in samples from those sources; N.A., no data available; —, never detected above the quantification limit of 0.05 μg/L.

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