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Identification of antimicrobial resistant bacteria in rivers: Insights into the cultivation bias

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ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form
16 April 2013
Accepted 20 May 2013
Available online 13 June 2013

Keywords:
Sewage polluted river
Antimicrobials
Antimicrobial resistance
Bacterial community composition

ABSTRACT

In the present study, the antimicrobial resistant (AR) bacteria were quantified and identified in different river samples using in parallel a culture-based approach and a culture-independent one. The objective was to evaluate the importance of the cultivation bias when studying antimicrobial resistance among environmental bacteria. Three different river samples covering a gradient of anthropic influence were tested and three different antimicrobial compounds were used as selective agents: amoxicillin, tetracycline and sulfamethoxazole. From a quantitative point of view, our results highlight the importance of the culture media used, as for the same sample and the same selective agent significant differences were observed in the counts of culturable AR bacteria depending on the culture media used. The identification of AR bacteria through culture or culture-independent methods put on evidence AR bacterial communities that differ dramatically: *γ-proteobacteria* and more specifically *Aeromonadaceae* dominated among the isolates while *β-proteobacteria* (*Comamonadaceae*), dominated among the sequences obtained without culture. Altogether these results highlight the necessity to develop a methodological consensus preferably without culture, to approach this important topic in the coming years.

1. Introduction

The occurrence of antimicrobial resistant (AR) bacteria is a major concern in clinical microbiology and the research on antimicrobial resistance of bacteria over the last fifty years was mainly focused on pathogenic bacteria. Nevertheless, it is now well documented that the development of AR had been going on in nature long before antimicrobials were used for medical and veterinary purposes (Aminov, 2009). The existence among the environmental bacteria of a reservoir of AR mechanisms (environmental resistome) (D'Costa et al., 2006), still largely unknown, and the potential transfer to pathogenic bacteria represent a real health issue for the future generations.

Besides, human activity has markedly enhanced the evolution and distribution of resistant bacteria in hospitals and in the natural environment; the increase of the occurrence of AR bacteria has raised the attention during the last ten years on the use and disposal of pharmaceuticals and especially antimicrobial compounds (Kummerer, 2003). The presence of

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0043-1354/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.watres.2013.05.036

antimicrobials in the environment and the associated selective pressure, could also contribute to the spread of resistance genes among environmental bacteria (Baquero et al., 2009) especially among emergent pathogens, which represent a major challenge for public health in the modern world (Sharma et al., 2003).

In general, aquatic systems and specially freshwaters, with their high and diverse bacterial load and the anthropogenic impact on them, are not only sinks of AR mechanisms but have an important ecological and evolutionary role in driving the persistence, emergence and spread of AR bacteria (Taylor et al., 2011). Antimicrobial concentrations sufficiently high to represent an ecological threat have already been reported in different rivers worldwide (Managaki et al., 2007).

Most of the research reports dealing with the identification of AR bacteria in aquatic systems were based on isolates and the same types of opportunistic bacteria were reported everywhere (i.e.: *Pseudomonas*, *Flavobacterium*, *Xanthomonas*, *Aeromonas*, *Acinetobacter Klebsiella*, *Serratia*, *Stenotrophomonas*) (De Souza et al., 2006; Blasco et al., 2008). As the majority of bacterial species in aquatic environments remain "not yet cultivated", these culture-based approaches will not allow a complete view of the diversity of AR bacteria in environmental samples.

The few studies, using culture-independent methods, consider the tolerance patterns of microbial communities without differentiation among the resistant phylotypes (Stepanauskas et al., 2005) or search for resistance genes independently of which organism is carrying such genes (Pruden et al., 2012). Our previous work (Garcia-Armisen et al., 2011) suggests that one might expect that a culture-independent approach allowing the phylogenetic identification of AR bacteria could reveal other dominant groups or strains than those identify by culture-based methods. A very recent article (Li et al., 2011) describes the composition of the bacterial community in a river strongly polluted by antimicrobials, but still, their methodology based on clone libraries described the whole community in such a site; but, using this approach, it was not possible to know which of these phylotypes were effectively AR, or if the identified phylotypes were still viable in the analyzed samples. In addition, the estimation of the proportion of resistant bacteria presented in this paper was evaluated by a culture-based method on a rich agar medium, so the proportions of AR bacteria published were still biased.

As there is not a consensus about the methodology to approach this important topic, the different reports published are difficult to compare and finally little is know about the identity of autochthonous aquatic bacteria carrying AR determinant or about the changes induced by anthropic activity into the AR patterns of environmental bacteria.

The goal of the present study is to quantify and identify the AR bacterial phylotypes in different freshwater samples using in parallel a culture-based approach and a culture-independent one and to analyze the differences in AR bacterial composition due to the methodology. The influence of the type of culture media used and the differences between the tested antimicrobials were evaluated. For the culture-independent method, the viability criterion was the membrane integrity: the proportion and the identity of all the bacteria that conserve their membrane integrity were evaluated after a 24 h incubation of the water sample with the selected antimicrobials.

2. Materials and methods

2.1. Experimental strategy

2.1.1. Samples collection

The three water samples used in this study were collected in the Seine watershed (France) in October 2010. This watershed is characterized by a high population density, intense industrial activity, and intensive agriculture. In the Parisian area, the Seine River receives the treated effluents of 10 millions inhabitants of the conurbation. Three samples were used with the objective to cover different degrees of anthropogenic pollution: The first one was collected in a small forest stream (FS) of the Oise River (a tributary from the Seine River) watershed upstream from any wastewater outfall. The second one (SE) was sampled in the Seine River at Evry which is located 30 km upstream from Paris; at this station, the river is not yet impacted by the release of the treated wastewaters from Paris and its suburbs. The third sample was collected in the Seine River at Conflans (SC), downstream from Paris; this sampling station is located 8 km downstream from the release of treated effluents of the large (1,700,000 $m^3\ d^{-1}$) Seine-Aval wastewater treatment plant. All samples were collected in sterile 2 L bottles, kept at 4 °C and analyzed within 12 h.

2.1.2. Samples analysis

For all the samples, temperature, pH, dissolved oxygen and conductivity were measured on the field with a probe: HQ40d portable meter (HACH). Samples were analyzed for total suspended solids and chemical oxygen demand (COD) using standard methods 2540D and 5220 respectively. *E. coli* concentrations were determined by plate counts on Chromocult Coliform Agar (CCA) (Merck KGaA, Darmstadt, Germany); colonies were enumerated after 24 h of incubation at 36 °C.

The concentrations of six antimicrobials (amoxicillin (AMX), tetracycline (TET), sulfamethoxazole (SMX), erythromycin (ERY), norfloxacin (NOR) and vancomycin (VAN)) were determined by an automated on-line solid phase extraction (SPE)–liquid chromatography–tandem mass spectrometry (LC–MS/MS) as described in Dinh et al. (2011). Limits of detection were in the range 1.7–45.6 ng l^{-1} for the different antimicrobials analyzed (Table 1).

2.1.3. Experimental design

To study the antimicrobial resistant bacteria, two parallel approaches were performed: one based on cultivation and the other that is culture-independent. For both approaches, resistances to amoxicillin (AMX), tetracycline (TET) and sulfamethoxazole (SMX) were tested. AMX and TET were chosen because they are among the most used antimicrobials and because they belong to different families and have different targets: TET is a protein synthesis inhibitor and AMX is an inhibitor of the synthesis of the bacterial peptidoglycan cell wall. SMX was selected because recent reports highlight a high concentration of this antibiotic in the Seine River downstream from Paris (Tamtam et al., 2008).