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Antibiotic resistance genes in bacterial and bacteriophage fractions of Tunisian and Spanish wastewaters as markers to compare the antibiotic resistance patterns in each population

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ABSTRACT

The emergence and increased prevalence of antibiotic resistance genes (ARGs) in the environment may pose a serious global health concern. This study evaluates the abundance of several ARGs in bacterial and bacteriophage DNA via real-time qPCR in samples from five different sampling points in Tunisia; three wastewater treatment plants (WWTP 1, 2 and 3) and wastewater from two abattoirs slaughtering different animals. Results are compared with those obtained in the Barcelona area, in northeast Spain.

Eight ARGs were quantified by qPCR from total and phage DNA fraction from the samples. Three β-lactamases (bla_{TEM}, $bla_{CTX\text{-}M}$ cluster 1 and $bla_{CTX\text{-}M}$ cluster 9), two quinolone resistance genes (*qnrA* and *qnrS*), the *mecA* gene that confers resistance to methicillin in *Staphylococcus aureus*, the emerging *armA* gene, conferring resistance to aminoglycosides and *sul1*, the most extended gene conferring resistance to sulfonamides, were evaluated.

Sul1 and bla_{TEM} were the most prevalent ARGs detected at all five Tunisian sampling points, similarly with the observations in Barcelona. $bla_{CTX\text{-}M\text{-}9}$ was more prevalent than $bla_{CTX\text{-}M\text{-}1}$ both in bacterial and DNA within phage particles in all samples analysed. *mecA* and *armA* were almost absent in Tunisian waters from human or animal origin in contrast with Barcelona that showed a medium prevalence. *qnrA* was more prevalent than *qnrS* in bacterial and phage DNA from all sampling points.

In conclusion, our study shows that ARGs are found in the bacterial and is reflected in the phage DNA fraction of human and animal wastewaters. The densities of each ARGs vary depending on the ARGs shed by each population and is determined by the characteristics of each area. Thus, the evaluation of ARGs in wastewaters seems to be suitable as marker reflecting the antibiotic resistance patterns of a population.

1. Introduction

Antibiotic resistance, both in human and animal pathogens, has emerged since the introduction of antibiotics in anthropogenic environments, compromising public and animal health worldwide. Antimicrobial resistance is a major global health problem, but marked variations in the resistance profiles of bacterial pathogens are found between countries and different geographic areas.

The appearance of antibiotic resistances is modulated by events of co-option, mutation, recombination and/or horizontal gene transfer between strains and, once have occurred, are subjected to natural selection that allows the widespread dispersal of the strains due to current globalization. The spread of resistance varies both temporally and geographically (Klugman, 2002; Kumarasamy et al., 2010; Nübel et al., 2008; Robicsek et al., 2006; Rodriguez-Martinez et al., 2011). Consequently, there is a temporal progression in the abundance of the corresponding antibiotic resistance genes (ARGs) and their geographical distribution that allows the occurrence of ARGs to be used to analyse the patterns of antibiotic resistance of a given population (Hawkey and Jones, 2009; Paniagua et al., 2010), and could be useful to detect changes on these patterns. To date, studies of the distribution and epidemiology of resistance have mostly been based on, and hence biased towards, the study of pathogens isolated in the clinical environment.

However, it is well known that it is not only pathogens that evolve these ARGs or gene arrangements, but that human and animal commensals also play an important role in the appearance and spread of these genes (Rolain, 2013; Salyers et al., 2004; Sommer et al., 2009;

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Wasyl et al., 2013). Therefore, microbial populations associated with humans and animals should be a good setting for searching for information about the epidemiology of resistance. In addition, the microbiological composition of some wastewaters, such as municipal sewage or abattoir wastewaters, is mostly due to human and animal gut microbiomes. It is thus likely that the ARGs found in bacterial populations in wastewaters represent the ARGs dominating in environments in contact with man-made antibiotics. The presence of ARGs and resistant bacteria in wastewaters has been widely reported (Novo et al., 2013; Rizzo et al., 2013; Schwartz et al., 2003; Szczepanowski et al., 2009; Tennstedt et al., 2003; Volkmann et al., 2004).

The potential for detecting these ARGs by genomic techniques makes the characterization of the resistome, total or partial, of the microbial populations present in wastewaters feasible. These sorts of studies are typically focused on bacterial DNA. However, the virome, mostly constituted by bacteriophages, has also recently been reported to contain abundant and diverse ARGs in the human gut (Minot et al., 2011; Quirós et al., 2014) and in human and animal wastewater (Colomer-Lluch et al., 2011a, 2011b; Muniesa et al., 2004; Parsley et al., 2010). Moreover, the fact that genomic studies can be performed independently of culture allows the use of archival sampling. So, although the finding of resistance determinants in a bacterium might not correspond to its phenotypic resistance, the presence of ARGs detected and quantified by molecular techniques will certainly provide relevant information regarding the ARGs circulating in a given environment or geographical area.

This study was conducted using archived and fresh samples from raw sewage entering three municipal and two abattoir wastewater treatment plants (WWTPs) in Tunisia, and aimed to evaluate the abundance of several ARGs in bacterial and bacteriophage DNA via real-time qPCR and to compare this with results of the Barcelona area in Spain.

The following ARGs were studied: three β-lactamases: bla_{TEM}, which includes more than 145 variants, $bla_{CTX\text{-}M}$ cluster 1, which includes variants CTX-M-1, 3, 10, 11 and 15, and $bla_{CTX\text{-}M}$ cluster 9, which includes variants CTX-M-9, 13, 14, 16 to 19, 21 and 27; two quinolone resistance genes: *qnrA* (including variants *qnrA1* to *qnrA7*) and *qnrS* (including variants *qnrS1* to *qnrS6*); the *mecA* gene, which confers resistance to methicillin in *Staphylococcus aureus* (MRSA); *armA* conferring resistance to aminoglycosides and *sul1* to sulfonamides. Quantification was performed using previously described methodology (Colomer-Lluch et al., 2011a, 2011b) from the same sample volume and by the same analyst, allowing us to compare the distribution of the ARGs in two different areas that differ in socio-economic and cultural characteristics, climate (especially in the southern location sampled in Tunisia) and geographic background.

These ARGs were selected because bla_{TEM} and $bla_{CTX\text{-}M}$ have been reported to be the most widely distributed extended spectrum β-lactamases (ESBLs) worldwide among Gram-negative pathogenic bacteria, while CTX-M-9 and CTX-M-14 are the most frequent $bla_{CTX\text{-}M}$ groups in animal isolates (Coque et al., 2008; Patterson, 2003; Rodriguez-Baño et al., 2008). *mecA* was selected because it confers resistance in Gram positive bacteria that showed a high prevalence in our geographical area (Colomer-Lluch et al., 2011a, 2011b). With regard the quinolones and fluoroquinolones, these are antimicrobials that are commonly used in clinical and veterinary medicine. Resistance to quinolones is dramatically increasing and has been described both in chromosome and acquired mobile genetic elements (MGEs) such as plasmids and in phage DNA in wastewater (Colomer-Lluch et al., 2014 Hooper, 2001; Paterson, 2006). *armA* was included because is highly prevalent in *Enterobacteriaceae* and it is considered an emerging gene spreading worldwide (González-Zorn et al., 2005). Finally, a new qPCR assay was developed in this study to evaluate *sul1*, the first described and most prevalent of the three *sul* genes conferring resistance to sulfonamides in raw sewage (Du et al., 2014; Wang et al., 2013).

2. Materials and methods

2.1. Sampling settings

Samples from Tunisia comprise incoming raw sewage to WWTPs sampled in three locations between 2011 and 2014. The first location was a WWTP treating water equivalent to 20,000 inhabitants corresponding to three towns in a pre-desert area (very warm in summer and low yearly rainfall) in South Tunisia (WWTP 1) with contribution of waste from tourist facilities. The second and third locations were municipal medium-load WWTP in a metropolitan area in North Tunisia (WWTP 2 and WWTP 3), the origin of its treated influents being domestic. Concerning animal wastewater samples, these were collected from one abattoir slaughtering mostly sheep and to lesser extent cattle (SLH1). SLH samples were collected in two different periods and were considered as two different set of samples. The first one corresponds to samples collected in 2011 (SLH1.1) and the second in 2014 (SLH1.2) (Fig. 1). Samples from Spain comprise incoming raw sewage to a WWTP treating water equivalent to 500,000 inhabitants in the area of Barcelona between 2011 and 2014 and wastewater from farms from different animals (cattle, poultry and pigs), from the same origin and showed similar microbiological contents as previously described (Colomer-Lluch et al., 2011b)

The fecal load of the samples studied as measured by the concentration of somatic coliphages (Jebri et al., 2012) was similar for samples from Tunisia and Spain showing fecal loads as reported in the Barcelona area for these types of sample (Muniesa et al., 2012). The samples were

Fig. 1. Geographic location of the five Tunisian sampling points: WWTP 1–3 and the two sets of slaughterhouses SLH 1.1 and SLH 1.2. Dotted line shows Tunis metropolitan area.