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Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia☆

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ABSTRACT

Antimicrobial resistance is a growing and significant threat to global public health, requiring better understanding of the sources and mechanisms involved in its emergence and spread. We investigated the abundance of antibiotic resistance genes (ARGs) before and after treatment in five wastewater treatment plants (WWTPs) located in different areas of the Monastir Governorate (Tunisia). Three of these WWTPs (Frina, Sahline and Zaouiet) use a conventional activated sludge process as secondary treatment, whereas the WWTP located in Beni Hassen applies an ultraviolet disinfection step after the activated sludge process and the WWTP located in Moknine treats wastewater using naturally aerated lagoons as a secondary treatment process. The abundance of six ARGs (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnrA*, *qnrS*, *sul* I and *ermB*) and the class 1 integron-integrase gene (*intI1*) were determined by quantitative PCR. All ARGs and the *intI1* gene were detected in the wastewater samples, except the *bla*$_{CTX-M}$ gene, which was not detected in both influent and effluent samples from Sahline and Beni Hassen WWTPs, and the *qnrS* gene, which was not detected neither in the WWTP influent in Moknine nor in the WWTP effluent in Beni Hassen. Although the relative concentration of ARGs was generally found to be similar between samples collected before and after the wastewater treatment, the abundance of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *qnrS* genes was higher in the effluent of the Frina WWTP which, unlike other WWTPs, not only receives domestic or industrial sewage but also untreated hospital waste. To the best of our knowledge, this study quantified for the first time the abundance of ARGs in different Tunisian WWTPs, and the results agree with previous studies suggesting that conventional wastewater treatment does not efficiently reduce ARGs. Therefore, these findings could be useful to improve the design or operation of WWTPs.

1. Introduction

Antibiotics have reduced mortality and morbidity from most infectious diseases but their extensive use over the past 75 years has made almost all bacterial pathogens resistant to antibiotics commonly used to treat them (Laxminarayan et al., 2013). The World Health Organization (WHO) has described antibiotic resistance as one of the greatest threats to human health. According to the recent report from the UK Review on Antimicrobial Resistance (AMR), 700,000 people die every year from infections due to resistant organisms, with numbers expected to rise to over 10 million by 2050 (http://amr-review.org).

Recent studies have suggested that antibiotic resistance found in bacterial pathogens largely originated from environmental settings (Wright, 2010; Pruden, 2014). In fact, a mixture of pollutants (*e.g.* antibiotics and heavy metals) and resistant bacteria reaches the environment through treated or untreated wastewater discharges, livestock operations, aquaculture, and industry, which may exert a selection pressure that eventually promotes the emergence, maintenance, and spread of antibiotic resistance in environmental bacteria (Martinez, 2009; Cabello et al., 2013; Berendonk et al., 2015).

Wastewater treatment plants (WWTPs) are considered hotspots for stimulating and promoting the dissemination of antibiotic

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resistance genes (ARGs). Conventional wastewater treatment processes, such as activated sludge and anaerobic digestion, provide an ideal setting for horizontal gene transfer, which is responsible, although not exclusively, for increasing the incidence and prevalence of antibiotic-resistant infections (Warnes et al., 2012). Horizontal gene transfer is mediated by mobile genetic elements, including plasmids, bacteriophages, transposons, and integron-associated gene cassettes, which play a major role in the short term adaptation of bacteria to local conditions and their evolution in the long term (Frost et al., 2005; Marti et al., 2014).

Despite antibiotic resistance has extensively been studied in bacterial pathogens, the expansion of this phenomenon has not been fully explored in WWTPs from developing countries. In Tunisia, for instance, several surveillance studies have been conducted to determine the prevalence of ARGs, especially genes conferring resistance to β-lactams and fluoroquinolones, in bacterial isolates from hospital- and community-acquired infections (Poirel et al., 2008; Mansour et al., 2009; Dahmen et al., 2010; Chouchani et al., 2011; Mansour et al., 2015). In contrast, studies on the diversity and abundance of ARGs in Tunisian WWTPs are lacking. The increasing use of treated urban wastewater for different purposes, such as agricultural and landscape irrigation, have compelled to develop guidelines to provide guidance and criteria on the level of quality required for wastewater discharge and reuse (Bahri and Brissaud, 1996; UNEP, 2005). These guidelines, however, do not take into account neither the potential presence of antibiotic-resistant bacteria and resistance genes in reclaimed water nor their concentration thresholds. Considering that WWTP effluents are either directly released into streams, rivers, and coastal water bodies, or reused in crop irrigation, it becomes necessary to examine to what extent WWTPs contribute to the spread of antibiotic resistance in developing countries such as Tunisia. Previous studies conducted in WWTPs from other regions, including developed countries, have demonstrated that their effluents can increase the prevalence of ARGs in the receiving environment (Storteboom et al., 2010; LaPara et al., 2011; Marti et al., 2013; Berglund et al., 2015), although few of them have explored the efficiency of wastewater treatments in removing ARGs (Biswal et al., 2014a; Di Cesare et al., 2016). Accordingly, our working hypothesis was that effluents from selected WWTP were enriched in ARGs despite differences in the treatment process, which ranged from conventional secondary treatment (*i.e.* activated sludge), ultraviolet (UV) disinfection step after activated sludge and naturally aerated lagoons.

For this purpose, we measured the concentration of several ARGs in five WWTPs located in the Monastir Governorate, central-eastern Tunisia, to assess the efficiency of wastewater treatment processes and to evaluate the potential contribution of these WWTPs on the dissemination of ARGs into the environment. The selection of genes was done according to their clinical and environmental relevance, including genes conferring resistance to the main antibiotic families used to treat bacterial infections, namely: β-lactams (bla_{TEM} and bla_{CTX-M}), fluoroquinolones (*qnrA* and *qnrS*), macrolides (*ermB*), and sulfonamides (*sul* I). Additionally, we have also determined the abundance of the class 1 integron-integrase gene (*intI1*) as proxy for both gene transfer and anthropogenic pollution (Gillings et al., 2015).

2. Materials and methods

2.1. Study sites and sampling

Five municipal WWTPs located in the Monastir Governorate, central-eastern Tunisia, were selected for this study (Fig. 1). These WWTPs serve between 13,488 and 165,184 people and three of them provide secondary treatment through conventional activated sludge processes but with different capacities and type of sewage (Table 1). Moreover, the WWTP located in the area of Beni Hassen uses activated sludge and a tertiary UV disinfection treatment, whereas the WWTP located in the area of Moknine treats wastewater using naturally aerated lagoons as secondary treatment process. In all studied WWTPs, the sewage influent undergoes a preliminary treatment to remove large solids. At each WWTP, influent and effluent water samples (150 ml) were collected in triplicate and stored at 4 °C in a portable icebox until arrival at the laboratory. Upon arrival, water samples were filtered through 0.22-μm-pore-size membranes (Millipore; Billerica, MA, USA) and filters were then stored at −30 °C until processing. The collected biomass was resuspended in lysis buffer (20 mM Tris·Cl [pH 8.0], 2 mM EDTA and 1.2% Triton X-100) and treated with lysozyme (20 mg/ml) for 1 h at 37 °C. Genomic DNA was then extracted using the DNeasy Blood & Tissue Kit (Qiagen; Valencia, CA, USA), and the concentration was determined using NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA). All DNA extracts were stored at −20 °C until analysis.

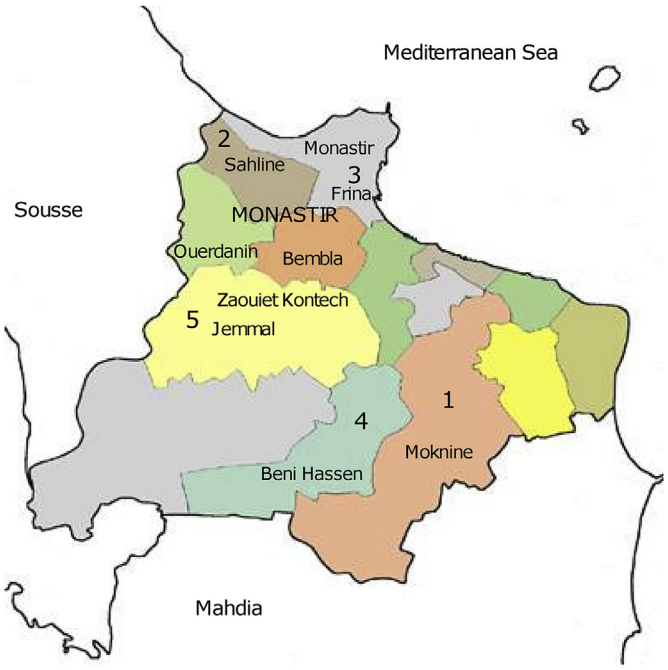

Fig. 1. Numbers in the map indicate the geographical location of the studied wastewater treatment plants (WWTPs).

2.2. Quantification of ARGs

Copy numbers of the *intI1* gene and selected ARGs (bla_{CTX-M}, bla_{TEM}, *qnrA*, *qnrS*, *sul* I and *ermB*) were determined by qPCR as previously described (Marti et al., 2013; Proia et al., 2016). Briefly, all qPCR assays were carried out in a MX3005P system (Agilent Technologies; Santa Clara, CA, USA) and using 10 ng μl^{-1} of template DNA in a final volume of 30 μl, containing 200 nM each forward and reverse primer and 2 × Brilliant III Ultra-Fast QPCR master mix (Agilent Technologies), except for the bla_{TEM} gene that was amplified using the SYBR Green master mix (Applied Biosystems; Carlsbad, CA, USA). The sequence of primers and PCR conditions for each gene are shown in Table S1. Copy number of the bacterial 16S rRNA gene was also quantified according to the previously described conditions (Maeda et al., 2003) and used as proxy for bacterial abundance and for data normalization. In all cases, a