# Virulence and plasmidic resistance determinants of *Escherichia coli* isolated from municipal and hospital wastewater treatment plants

Vera Calhau, Catarina Mendes, Angelina Pena, Nuno Mendonça and Gabriela Jorge Da Silva

## ABSTRACT

*Escherichia coli* is simultaneously an indicator of water contamination and a human pathogen. This study aimed to characterize the virulence and resistance of *E. coli* from municipal and hospital wastewater treatment plants (WWTPs) in central Portugal. From a total of 193 isolates showing reduced susceptibility to cefotaxime and/or nalidixic acid, 20 *E. coli* with genetically distinct fingerprint profiles were selected and characterized. Resistance to antimicrobials was determined using the disc diffusion method. Extended spectrum β-lactamase and plasmid-mediated quinolone resistance genes, phylogroups, pathogenicity islands (PAIs) and virulence genes were screened by polymerase chain reaction (PCR). CTX-M producers were typed by multilocus sequence typing. Resistance to beta-lactams was associated with the presence of $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}32}$. Plasmid-mediated quinolone resistance was associated with *qnr*A, *qnr*S and *aac(6′)-Ib-cr*. Aminoglycoside resistance and multidrug-resistant phenotypes were also detected. PAI $IV_{536}$, PAI $II_{CFT073}$, PAI $II_{536}$ and PAI $I_{CFT073}$, and uropathogenic genes *iut*A, *papAH* and *sfa/foc* were detected. With regard to the clinical ST131 clone, it carried $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}type}$, *qnrS* and *aac(6′)-Ib-cr*; IncF and IncP plasmids, and virulence factors PAI $IV_{536}$, PAI $I_{CFT073}$, PAI $II_{CFT073}$, *iutA, sfa/foc* and *papAH* were identified in the effluent of a hospital plant. WWTPs contribute to the dissemination of virulent and resistant bacteria in water ecosystems, constituting an environmental and public health risk.

**Key words** | *Escherichia coli*, phylogeny, plasmidic resistance determinants, virulence factors, WWTP

**Vera Calhau**
**Catarina Mendes**
**Angelina Pena**
**Nuno Mendonça**
**Gabriela Jorge Da Silva** (corresponding author)
Faculty of Pharmacy,
University of Coimbra, Health Sciences Campus,
Azinhaga de Santa Comba,
3000-548 Coimbra,
Portugal
E-mail: *gjsilva@ci.uc.pt*

**Vera Calhau**
**Nuno Mendonça**
**Gabriela Jorge Da Silva**
Center of Neurosciences and Cell Biology,
University of Coimbra,
Coimbra,
Portugal

## INTRODUCTION

*Escherichia coli* is simultaneously a biological indicator of water treatment safety and an important human pathogen responsible for several diseases (Edberg *et al.* 2000; Kaper *et al.* 2004). *E. coli* presents several virulence and antimicrobial resistance genes which contribute to its success as a human pathogen (Pitout 2012). These genes may be disseminated by mobile genetic elements such as pathogenicity islands (PAIs), carriers of virulence factors, or plasmids with genes coding for both resistance and virulence determinants (Hacker *et al.* 1997; Carattoli 2009). Water constitutes a good matrix for the lateral transfer of mobile genetic elements (Taylor *et al.* 2011), which are responsible for the dissemination of virulence or resistance traits between bacteria from different sources, contributing to the modification of the natural bacterial ecosystems (Baquero *et al.* 2008).

Currently, an inverse relationship between antimicrobial resistance and virulence has been the consensus (Moreno *et al.* 2006). However, recently it has been shown that these two features may co-exist in the same genotype perpetuating the bacterial lineage and highlighting concern because of its dissemination (Dolejska *et al.* 2011; Colomer-Lluch *et al.* 2013).

Wastewater treatment plants (WWTPs) are designed to significantly reduce the biological contamination of water.

doi: 10.2166/wh.2014.327

Nevertheless, studies report resistant bacteria in effluents of treated water, and suggest that the conditions in WWTPs favour the proliferation of antibiotic-resistant bacteria and the exchange of genetic elements (Moura *et al.* 2007; Dolejska *et al.* 2011; Korzeniewska *et al.* 2013). The emergence and dissemination of antimicrobial-resistant bacteria has led to increasing concerns about potential environmental and public health risks. Moreover, the carriage of specific virulence genes, especially those located in mobile genetic elements, are important to evaluate the public health risks.

The main objectives of this study were to characterize the virulence and antimicrobial resistance profiles of *E. coli* collected in waters from municipal and hospital WWTPs from central Portugal and to screen for the presence of mobile genetic elements.

## MATERIALS AND METHODS

### Bacterial isolates

Between April and May 2011, water samples were collected from four hospitals and three municipal WWTPs located in the central region of Portugal:

- University hospital: reference hospital for the central region of Portugal. A large hospital with 1,456 beds, with an extended set of medical specialties and clinical services, as well as a centre of research, serving a population of approximately 430,000 inhabitants.
- General hospital: medium-sized hospital with 13 main wards and 350 beds. It serves a population of approximately 369,000 inhabitants.
- Pediatric hospital: small reference hospital in central Portugal that supports paediatric units. It is composed of nine main wards and 110 beds serving a population of about 90,000 inhabitants.
- Maternity: small hospital with 96 beds and three main wards – gynaecology, obstetrics and neonatology, not including the baby unit. It serves a population of approximately 507,000 women.
- Municipal WWTP1: serves a 14,000 population equivalent.
- Municipal WWTP2: serves a 213,000 population equivalent. It receives urban wastewaters that include domestic wastewaters and hospital effluents (namely from the four mentioned hospitals).
- Municipal WWTP3: serves a 1,500 population equivalent.

Municipal WWTP sampling was performed at the entrance and exit of the station on two occasions and hospital samples were collected on three different dates at the exit of the station. Wastewater samples (250 mL) were collected in amber glass bottles and further vacuum filtered through 1.0 μm glass microfibre filters (GF/C, Whatman, UK), followed by 0.45 μm nylon membrane filters (Whatman, UK). The filters were placed in MacConkey Agar supplemented with 0.5 mg/L of cefotaxime or 10 mg/L of nalidixic acid. A bacterial suspension was prepared with the inoculum and cultured in MacConkey Agar. A maximum of eight presumptive colonies of *E. coli* per plate were further cultured in Eosin Methylene Blue Agar, and lactose fermenter colonies with a green metallic sheen were selected. The citrate test was used to distinguish *E. coli* from *Citrobacter* spp. The identification was confirmed using a polymerase chain reaction (PCR)-based technique with specific primers set targeting *uid*A gene (Heijnen & Medema 2006).

The genetic relationship was evaluated by BOX-PCR (Versalovic *et al.* 1994), and only non-duplicate isolates were further analysed.

### Susceptibility testing and phenotypic extended spectrum β-lactamase detection

The antimicrobial susceptibility profiles for ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), nalidixic acid (30 μg), ciprofloxacin (10 μg) and gentamicin (10 μg) were determined using a disc diffusion test. Extended spectrum β-lactamase producers were detected with the double disc synergy test (Jarlier *et al.* 1988). The methods were performed and the results were interpreted based on the Clinical and Laboratory Standards Institute guidelines (CLSI 2010). Multidrug resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.* 2012).

### Antimicrobial resistance determinants detection

The $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$ genes coding for β-lactamases and plasmid-mediated quinolone resistance (PMQR) determinants *qnrA*, *B* and *S*, and *qepA* were screened with specific primers by PCR (Cattoir *et al.* 2007; Mendonça *et al.* 2007; Ma *et al.* 2009). For the samples with a positive result for the screening of $bla_{CTX\text{-}M}$, the full gene was further amplified using previously described primers (Conceição *et al.* 2005) and amplicons were purified with ExoSAP-IT (Affymetrix, USB products). The whole genes were sequenced at Macrogen, Amsterdam, The Netherlands.

*aac(6′)-Ib* was screened by PCR and isolates positive for the *aac(6′)-lb* gene were further digested with BtsCI enzyme (New England Biolabs) to identify *aac(6′)-lb-cr* which lacks the BtsCI restriction site present in the wild-type gene (Park *et al.* 2006).

### Plasmid replicon typing

Plasmid replicon identification was performed according to the PCR-based replicon typing scheme (Carattoli *et al.* 2005), detecting the main replicon families in *Enterobacteriaceae*.

### Detection of PAIs and other virulence markers

PAI markers were screened according to the Bronowski *et al.* (2008) scheme, based on the technique first described by Sabaté *et al.* (2006). This method allows the detection of eight PAIs, encoding several virulence determinants: PAI $I_{536}$, PAI $II_{536}$, PAI $III_{536}$, PAI $IV_{536}$, PAI $I_{J96}$, PAI $II_{J96}$, PAI $I_{CFT073}$, and finally PAI $II_{CFT073}$ (Sabaté *et al.* 2006).

Other virulence genes that may be present in extraintestinal *E. coli* (EXPEC) such as *papAH, papC* (P fimbriae structural subunit and assembly), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *iutA* (aerobactin receptor), *kpsM II* (group 2 capsules) and *cnf1* (cytotoxic necrotizing factor 1) were screened by PCR (Johnson & Stell 2000), as well as the enterohaemorrhagic *E. coli* associated virulence genes *eaeA* (intimin), *hlyA* (pore-forming cytolysin), *stx* 1 and 2 (shiga-like toxins) (Ram *et al.* 2008).

### Phylogenetic analysis

The determination of *E. coli* major phylogroups (A, B1, B2 and D) was performed with a PCR-multiplex detecting *chuA*, *yjaA* and DNA fragment tspE4.C2 genes (Clermont *et al.* 2000; Mendonça *et al.* 2011).

### Multilocus sequence typing (MLST)

MLST of the CTX-M producers was performed based on the PCR amplification and sequencing of seven housekeeping genes, *adk, fumC, gyrB, icd, mdh, purA* and *recA,* according to the University College of Cork (Cork, Ireland) scheme for *E. coli (*http://mlst.ucc.ie/mlst/dbs/Ecoli).

## RESULTS

### Bacterial isolates

A total of 193 presumed *E. coli* with reduced susceptibility to cefotaxime and/or nalidixic acid were obtained from WWTPs. The majority of the isolates showed an identical genetic profile and only 20 isolates with distinct profiles were selected (non-duplicate isolates) and further characterized for resistance and virulence profiles (Table 1). Fourteen of the non-duplicate isolates were from municipal WWTPs, while the remaining six were recovered from hospital water samples. The municipal isolates were recovered from WWTP2 ($n = 7$), followed by WWTP3 ($n = 4$) and WWTP1 ($n = 3$). Isolates W4 and W12 were detected in both the influent and effluent of the respective WWTPs, and in addition W12 isolate was detected on two different sampling occasions. From the hospital WWTPs, three strains were recovered from the general hospital, two from the maternity hospital and one from the university hospital. *E. coli* isolates with reduced susceptibility to CTX or NAL were not detected in the outflow of the paediatric hospital.

### Resistance profile characterization

The majority of the isolates were resistant to nalidixic acid (85%), followed by resistance to ampicillin (50%), amoxicillin-clavulanic acid (35%), cefoxitin (35%), cefotaxime (35%),

**Table 1** | Distribution of strains and characterization of phylogeny, virulence and resistance determinants and plasmid incompatibility groups

| WWTP | Strain | Collection date (day/month) | Sampling | Phylogroup | Virulence determinants | Resistance profile | Plasmidic resistance determinants | Replicon type | ST |
|---|---|---|---|---|---|---|---|---|---|
| **Hospital WWTP** | | | | | | | | | |
| Maternity | W1 | 19/4 | Outflow | A | PAI $IV_{536}$, *iut*A | NAL | – | F, FIA, FIB,K, I1/Iϒ, P | ND |
| | W2 | 9/5 | Outflow | D | *iut*A | AMP, NAL | $bla_{TEM}$ | I1/Iϒ, P | ND |
| University | W3 | 18/4 | Outflow | B2 | PAI $IV_{536}$, PAI $I_{CFT073}$, PAI $II_{CFT073}$, *iut*A, *sfa/foc, papAH* | AMP,CAZ, CTX, CN, NAL, CIP | $bla_{CTX-M-15}$, $bla_{TEM}$, *qnr*S, *aac(6′)-lb-cr* | F, FIB,P | ST131 |
| General | W16 | 19/4 | Outflow | D | *iut*A | AMP, FOX, CAZ, CTX, AMC, GEN, NAL, CIP | $bla_{TEM}$, *qnr*A | F | ND |
| | W17 | 19/4 | Outflow | A | – | AMP, FOX, CTX, NAL, CIP | $bla_{TEM}$ | – | ND |
| | W18 | 2/5 | Outflow | A | – | AMP, FOX, CAZ, CTX, AMC, NAL | $bla_{TEM}$ | – | ND |
| **Municipal WWTP** | | | | | | | | | ND |
| WWTP1 | W4 | 19/4 | Inflow/ Outflow | D | PAI $IV_{536}$ | – | – | – | ND |
| | W5 | 19/4 | Outflow | B1 | – | – | – | – | ND |
| | W6 | 19/4 | Outflow | B2 | PAI $II_{536}$, PAI $IV_{536}$, PAI $II_{CFT073}$, *iut*A | AMP, AMC | $bla_{TEM}$ | F, FIA, I1/Iϒ | ND |
| WWTP2 | W7 | 19/4 | Inflow | B2 | PAI $IV_{536}$, , PAI $II_{CFT073}$, *iut*A, *papAH* | AMP, GEN, NAL, CIP | $bla_{TEM}$, *qnr*S | F, FIA | ND |
| | W8 | 19/4 | Outflow | A | – | AMP, CAZ, CTX, NAL, CIP | $bla_{SHV}$ | I1/Iϒ | ND |
| | W9 | 19/4 | Outflow | B1 | PAI $IV_{536}$,*iut*A | AMP, FOX, CAZ, CTX, AMC, NAL,CIP | qnrA | F, FIA, FIB, K, I1/Iϒ,P | ND |
| | W10 | 19/4 | Outflow | B1 | PAI $IV_{536}$,*iut*A | NAL | – | F,K | ND |
| | W11 | 19/4 | Outflow | A | PAI$IV_{536}$, *iut*A | FOX, AMC, NAL | – | F | ND |
| | W12 | 19/4; 5/5 | Inflow/ Outflow | B2 | PAI $IV_{536}$, PAI $I_{CFT073}$, PAI $II_{CFT073}$, *iut*A, *eae*A | NAL | – | F | ND |
| | W13 | 5/5 | Inflow | D | PAI $IV_{536}$, *iut*A | FOX, NAL | – | P | ND |
| WWTP3 | W14 | 19/4 | Outflow | A | *iut*A | NAL | – | – | ND |
| | W15 | 5/5 | Inflow | A | PAI $IV_{536}$ | AMP, CTX, NAL, | $bla_{CTX-M-32}$ | F,K | ST34 |
| | W19 | 5/5 | Inflow | B2 | PAI $IV_{536}$,*iut*A | FOX, NAL | – | F, FIB | ND |
| | W20 | 5/5 | Inflow | B1 | PAI $IV_{536}$ | NAL | *qnr*A | K | ND |

AMP, ampicillin; CAZ, ceftazidime; FOX, cefoxitin, CTX, cefotaxime; GEN, gentamicin; CIP, ciprofloxacin; NAL, nalidixic acid; AMC, amoxicillin-clavulanic acid.
ND, not determined.

ciprofloxacin (30%), ceftazidime (25%) and gentamicin (15%). Strains W4 and W5 from municipal WWTP1 were susceptible to all the antibiotics tested, and strains W3 from the university hospital, strain W16 from the general hospital and strain W7 from the municipal WWTP2 were multidrug resistant. Among the antimicrobial resistance determinants screened, $bla_{TEM}$ was detected most ($n = 7$) followed by *qnr*A ($n = 3$), *qnr*S ($n = 2$) and $bla_{CTX\text{-}M}$ ($n = 2$). The studied resistance determinants were not detected in nine isolates.

Only two strains carried $bla_{CTX\text{-}M}$ genes: $bla_{CTX\text{-}M\text{-}15}$ (W3) collected from the university hospital outflow and $bla_{CTX\text{-}M\text{-}32}$ (W15) from the municipal WWTP3 inflow water. Isolate W3 was assigned by MLST to ST131 and isolate W15 to ST34. The strain W3 ST131 was multidrug resistant and showed the higher diversity of plasmidic determinants, carrying $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}type}$, *qnrS* and *aac(6′)-lb-cr*.

The main plasmid groups detected in *Enterobacteriaceae* family members were also investigated. Four plasmid groups: IncF, IncK, IncI1/Iγ and IncP were detected. IncF was the most prevalent group ($n = 11$) found in both hospital and municipal WWTPs waters, while in 25% of the isolates no plasmid was identified.

### Virulence profile description

The PAIs most frequently detected was PAI $IV_{536}$ ($n = 13$) followed by PAI $II_{CFT073}$ ($n = 5$), PAI $I_{CFT073}$ ($n = 2$) and PAI $II_{536}$ ($n = 1$). PAI $I_{536}$, PAI $III_{536}$, PAI $I_{J96}$ and PAI $II_{J96}$ were not detected. PAI $IV_{536}$ and PAI $II_{CFT073}$ were more prevalent in municipal isolates. PAI $II_{536}$ was exclusively detected in a strain from a municipal WWTP. Different combinations of PAIs were identified (Table 1).

Considering individual virulence genes, the most frequently detected was *iut*A ($n = 13$), followed by *papAH* ($n = 2$); more common among hospital isolates, *sfa/foc* and *eae*A were less prevalent, each of them being detected in one isolate, the former found in a hospital source and *eae*A detected in a municipal WWTP. The genes *afa/dra*, *kpsM II*, *cnf*, *hlyA*, *stx*1 and 2 were not detected. The most prevalent phylogenetic group was group A ($n = 7$) followed by B2 ($n = 5$), and finally B1 and D ($n = 4$, each). Strains from phylogroup B2 from both municipal and hospital WWTPs carried more virulence factors, including the ST131 isolate (Table 1). All the other isolates presented virulence determinants regardless of the phylogroup, with the exception of W17 and W8 from phylogroup A, and W5 from phylogroup B1.

## DISCUSSION

This study aimed to characterize the virulence and resistance profiles of *E. coli* selected from municipal and hospital WWTPs from a central region of Portugal, evaluating the possibility of environmental dissemination of pathogenic and/or resistant bacteria from these sources. Several studies indicate the potential dissemination of resistant and/or virulent bacteria from WWTPs into the environment (Jakobsen *et al.* 2008; Sabaté *et al.* 2008; Chagas *et al.* 2011; Dolejska *et al.* 2011; Colomer-Lluch *et al.* 2013; Biswal *et al.* 2014). Nonetheless, only one study concomitantly studied virulence factors and resistance determinants in hospital WWTPs (Jakobsen *et al.* 2008), and it only focused on gentamicin resistance determinants and in single virulence factors. Here, we extended the study to the identification of PAIs, clusters of virulence genes with the potential to be mobile.

*E. coli* strains showed resistance to important groups of antibiotics such as beta-lactams, quinolones and aminoglycosides, with multidrug resistance being detected in both municipal and hospital strains, indicating that WWTPs may be responsible for the introduction of multidrug-resistant bacteria into the environment. In the Portuguese Mondego river, where the effluents of the studied WWTPs are discharged, several types of antibiotics, including fluoroquinolones, were recently detected (Santos *et al.* 2013), which may exert a selective pressure in the dissemination of resistant bacteria in environmental waters (Kummerer & Henninger 2003). Plasmidic resistance determinants are important vehicles of transmission of resistance genes. Several resistance determinants were detected in this study, including $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}32}$, responsible for resistance to several beta-lactams, as well as PMQR genes, including *qnr*A, *qnr*S and *aac(6′)-Ib-cr*. Different beta-lactamase genes have already been detected, including $bla_{CTX\text{-}M\ group\ 1}$, $bla_{CTX\text{-}M\ group\ 9}$, $bla_{SHV}$ and

$bla_{TEM}$ genes, in hospital and municipal effluents (Korzeniewska & Harnisz 2013; Korzeniewska *et al.* 2013). In addition, CTX-M-15 and CTX-M-32 producers have already been detected in river waters in Portugal, with unknown origin, indicating that these determinants may be spreading among water systems (Tacão *et al.* 2012).

Several virulence factors responsible for enhancing the pathogenic potential of bacteria have been detected in *E. coli* (Johnson 1991; Johnson & Stell 2000), and some of them are clustered in PAIs, mobile genetic platforms capable of dissemination through horizontal gene transfer (Hacker *et al.* 1997). Virulence profiles were characterized in the isolates. Results show that PAI $IV_{536}$ was the most prevalent island, likewise in other studies performed in clinical samples and in water from several origins, but none of them from WWTPs (Sabaté *et al.* 2006; Mendonça *et al.* 2012). The association of PAI $IV_{536}$ to virulence is controversial, as some studies indicate that this island contributes to the virulence of EXPEC (Schubert *et al.* 2002) but other authors suggest that this is, rather, a fitness element (Oelschlaeger *et al.* 2002). Several uropathogenic *E. coli* (UPEC) virulence genes were identified in the isolates, including *iut*A, involved in the uptake of iron, *papAH* coding for P fimbriae associated with pyelonephritis (Kallenius *et al.* 1981; Dowling *et al.* 1987) and *sfa/foc* encoding S fimbriae/F1C fimbriae, involved in urinary infections, neonatal sepsis as well as meningitis (Antão *et al.* 2009). In addition, *eae*, usually detected in enteropathogenic and enterohaemorrhagic *E. coli* were also detected in a municipal isolate. This fact may be related to the possible association of animal farms to the municipal WWTP where W12 isolate was detected, as ruminants are known to be important reservoirs of *E. coli* carrying intimin gene (Blanco *et al.* 2005). This isolate also carried other virulence determinants and was detected in both the influent and effluent of the WWTP at different collection dates indicating that WWTPs are not only inefficient concerning the elimination of virulent bacteria, but are also contributing to the dissemination of strains carrying virulence-associated genes in the environment.

The phylogenetic background of the strains was studied as an indicator of the virulence potential of the isolates. *E. coli* strains have been grouped into four different phylogroups (A, B1, B2 and D) according to their virulence features. Virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D, while commensal strains belong to groups A and B1 (Clermont *et al.* 2000). Despite group A and B1 being considered less virulent, strains from these phylogroups harbouring virulence factors were detected in both municipal and hospital isolates. This observation may indicate that even the bacteria considered less virulent may be enhancing their virulent potential, possibly due to horizontal gene transfer of virulence traits.

In this study the international clone *E. coli* ST131 was detected in the effluent of a hospital. The ST131 isolate carried several pathogenic factors, including PAI $IV_{536}$, PAI $I_{CFT073}$, PAI $II_{CFT073}$, *iutA*, *sfa/foc* and *papAH* as well as resistance determinants $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM}$, *qnrS* and *aac(6′)-Ib-cr* and the IncF plasmid, a conjugative plasmid that can easily be spread to other bacteria, and is known for dissemination of $bla_{CTX\text{-}M\text{-}15}$ and *aac(6′)-Ib-cr* (Carattoli 2009; Partridge *et al.* 2011). ST131 displays both resistance and virulence features which contribute to the success of this international clone, which today is one of the most adapted and efficient human pathogens (Johnson *et al.* 2010a, b). This clone was previously detected in the effluent of a municipal WWTP in the Czech Republic (Dolejska *et al.* 2011) and in the influent of a WWTP in Catalonia, Spain (Colomer-Lluch *et al.* 2013). However, to our knowledge, this is the first finding in hospital WWTPs, highlighting the crucial need for monitoring the efficiency of hospital WWTPs.

The dissemination of bacteria carrying both resistance and virulence determinants, such as ST131, constitutes an important threat to public health and to the environment. Resistant or pathogenic isolates when in contact with autochthonous bacteria may be responsible for the dissemination of resistance and virulence determinants among natural ecosystems by horizontal gene transfer.

## CONCLUSIONS

WWTPs constitute a potential mechanism of propagation of resistant and pathogenic bacteria from sewage of diverse origins, into the environment, and may thus contribute to the environmental dissemination of virulence and resistance determinants which constitute an important public health concern.

## ACKNOWLEDGEMENTS

All authors contributed equally to this work. N. Mendonça was supported by a grant SFRH/BPD/45815/2008 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the European Society of Clinical Microbiology and Infectious Diseases 2010 Research Grant, the PEst-OE/SAU/UI0177/2014 from Fundação para a Ciência e Tecnologia, and by the Center for Pharmaceutical Studies, University of Coimbra, Portugal. No conflict of interest is declared.

## REFERENCES

Antão, E. M., Wieler, L. H. & Ewers, C. 2009 Adhesive threads of extraintestinal pathogenic *Escherichia coli*. *Gut Pathog.* **1** (1), 22.

Baquero, F., Martinez, J. L. & Canton, R. 2008 Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **19** (3), 260–265.

Biswal, B. K., Mazza, A., Masson, L., Gehr, R. & Frigon, D. 2014 Impact of wastewater treatment processes on antimicrobial resistance genes and their co-occurrence with virulence genes in *Escherichia coli*. *Water Res.* **50**, 245–253.

Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J. E., Dahbi, G., Blanco, J. & Stephan, R. 2005 Serotypes, intimin variants and other virulence factors of eae positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (*eae-eta2*). *BMC Microbiol.* **5**, 23.

Bronowski, C., Smith, S. L., Yokota, K., Corkill, J. E., Martin, H. M., Campbell, B. J., Rhodes, J. M., Hart, C. A. & Winstanley, C. 2008 A subset of mucosa-associated *Escherichia coli* isolates from patients with colon cancer, but not Crohn's disease, share pathogenicity islands with urinary pathogenic *E. coli*. *Microbiology* **154** (2), 571–583.

Carattoli, A. 2009 Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **53** (6), 2227–2238.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L. & Threlfall, E. J. 2005 Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* **63** (3), 219–228.

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J. & Nordmann, P. 2007 Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. *J. Antimicrob. Chemother.* **60** (2), 394–397.

Chagas, T. P., Seki, L. M., Cury, J. C., Oliveira, J. A., Dávila, A. M., Silva, D. M. & Asensi, M. D. 2011 Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *J. Appl. Microbiol.* **111** (3), 572–581.

Clermont, O., Bonacorsi, S. & Bingen, E. 2000 Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* **66** (10), 4555–4558.

CLSI 2010 Performance standards for antimicrobial susceptibility testing; twentieth informational supplement. CLSI document M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.

Colomer-Lluch, M., Mora, A., López, C., Mamani, R., Dahbi, G., Marzoa, J., Herrera, A., Viso, S., Blanco, J. E., Blanco, M., Alonso, M. P., Jofre, J., Muniesa, M. & Blanco, J. 2013 Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain. *J. Antimicrob. Chemother.* **68** (4), 758–765.

Conceição, T., Brízio, A., Duarte, A., Lito, L., Cristino, J. & Salgado, M. 2005 First description of CTX-M-15-producing *Klebsiella pneumoniae* in Portugal. *Antimicrob. Agents Chemother.* **49** (1), 477–478.

Dolejska, M., Frolkova, P., Florek, M., Jamborova, I., Purgertova, M., Kutilova, I., Cizek, A., Guenther, S. & Literak, I. 2011 CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and Klebsiella spp. isolates in municipal wastewater treatment plant effluents. *J. Antimicrob. Chemother.* **66** (12), 2784–2790.

Dowling, K. J., Roberts, J. A. & Kaack, M. B. 1987 P-fimbriated *Escherichia coli* urinary tract infection: a clinical correlation. *South. Med. J.* **80** (12), 1533–1536.

Edberg, S. C., Rice, E. W., Karlin, R. J. & Allen, M. J. 2000 *Escherichia coli*: the best biological drinking water indicator for public health protection. *Symp. Ser. Soc. Appl. Microbiol.* **29**, 106S–116S.

Hacker, J., Blum-Oehler, G., Mühldorfer, I. & Tschäpe, H. 1997 Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol. Microbiol.* **23** (6), 1089–1097.

Heijnen, L. & Medema, G. 2006 Quantitative detection of *E. coli*, *E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. *J. Water Health* **4** (4), 487–498.

Jakobsen, L., Sandvang, D., Hansen, L. H., Bagger-Skjot, L., Westh, H., Jorgensen, C., Hansen, D. S., Pedersen, B. M., Monnet, D. L., Frimodt-Moller, N., Sorensen, S. J. & Hammerum, A. M. 2008 Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. *Environ. Int.* **34** (1), 108–115.

Jarlier, V., Nicolas, M. H., Fournier, G. & Philippon, A. 1988 Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev. Infect. Dis.* **10**, 867–878.

Johnson, J. R. 1991 Virulence factors in *Escherichia coli* urinary tract infection. *Clin. Microbiol. Rev.* **4** (1), 80–128.

Johnson, J. R. & Stell, A. L. 2000 Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J. Infect. Dis.* **181** (1), 261–272.

Johnson, J. R., Johnston, B., Clabots, C., Kuskowski, M. A. & Castanheira, M. 2010a *Escherichia coli* sequence type ST131 as

the major cause of serious multidrug-resistant *E. coli* infections in the United States. *Clin. Infect. Dis.* **51** (3), 286–294.

Johnson, J. R., Johnston, B., Clabots, C., Kuskowski, M. A., Pendyala, S., Debroy, C., Nowicki, B. & Rice, J. 2010b *Escherichia coli* sequence type ST131 as an emerging fluoroquinolone-resistant uropathogen among renal transplant recipients. *Antimicrob. Agents Chemother.* **54** (1), 546–550.

Kallenius, G., Mollby, R., Svenson, S. B., Helin, I., Hultberg, H., Cedergren, B. & Winberg, J. 1981 Occurrence of P-fimbriated *Escherichia coli* in urinary tract infections. *Lancet* **2** (8260–8261), 1369–1372.

Kaper, J. B., Nataro, J. P. & Mobley, H. L. 2004 Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* **2** (2), 123–140.

Korzeniewska, E. & Harnisz, M. 2013 Beta-lactamase-producing *Enterobacteriaceae* in hospital effluents. *J. Environ. Manage.* **123**, 1–7.

Korzeniewska, E., Korzeniewska, A. & Harnisz, M. 2013 Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol. Environ. Saf.* **91**, 96–102.

Kummerer, K. & Henninger, A. 2003 Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin. Microbiol. Infect.* **9** (12), 1203–1214.

Ma, J., Zeng, Z., Chen, Z., Xu, X., Wang, X., Deng, Y., Lu, D., Huang, L., Zhang, Y., Liu, J. & Wang, M. 2009 High prevalence of plasmid-mediated quinolone resistance determinants *qnr, aac (6′)-Ib-cr*, and *qep*A among ceftiofur-resistant *Enterobacteriaceae* isolates from companion and food-producing animals. *Antimicrob. Agents Chemother.* **53** (2), 519–524.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T. & Monnet, D. L. 2012 Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **18** (3), 268–281.

Mendonça, N., Leitao, J., Manageiro, V., Ferreira, E. & Caniça, M. 2007 Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrob. Agents Chemother.* **51** (6), 1946–1955.

Mendonça, N., Calhau, V., Lin, T., Boaventura, L., Ribeiro, G. & Da Silva, G. J. 2011 Unusual genotype of a uropathogenic *Escherichia coli* strain assigned to the B2 phylogenetic group. *J. Clin. Microbiol.* **49** (8), 3105–3106.

Mendonça, N., Ramalho, J., Vieira, P. & Da Silva, G. J. 2012 Association of plasmid-mediated quinolone resistance and virulence markers in *Escherichia coli* isolated from water. *J. Water Health* **10** (2), 288–294.

Moreno, E., Prats, G., Sabaté, M., Pérez, T., Johnson, J. R. & Andreu, A. 2006 Quinolone, fluoroquinolone and trimethoprim/sulfamethoxazole resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli*. *J. Antimicrob. Chemother.* **57** (2), 204–211.

Moura, A., Henriques, I., Ribeiro, R. & Correia, A. 2007 Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J. Antimicrob. Chemother.* **60** (6), 1243–1250.

Oelschlaeger, T. A., Dobrindt, U. & Hacker, J. 2002 Pathogenicity islands of uropathogenic *E. coli* and the evolution of virulence. *Int. J. Antimicrob. Agents* **19** (6), 517–521.

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D. & Hooper, D. C. 2006 Prevalence in the United States of *aac′-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob. Agents Chemother.* **50** (11), 3953–3955.

Partridge, S. R., Zong, Z. & Iredell, J. R. 2011 Recombination in IS26 and Tn2 in the evolution of multiresistance regions carrying *bla*CTX-M-15 on conjugative IncF plasmids from *Escherichia coli*. *Antimicrob. Agents Chemother.* **55** (11), 4971–4978.

Pitout, J. D. 2012 Extraintestinal pathogenic *Escherichia coli*: A combination of virulence with antibiotic resistance. *Front. Microbiol.* **3**, 9.

Ram, S., Vajpayee, P. & Shanker, R. 2008 Contamination of potable water distribution systems by multiantimicrobial-resistant enterohemorrhagic *Escherichia coli*. *Environ. Health Perspect.* **116** (4), 448–452.

Sabaté, M., Moreno, E., Pérez, T., Andreu, A. & Prats, G. 2006 Pathogenicity island markers in commensal and uropathogenic *Escherichia coli* isolates. *Clin. Microbiol. Infect.* **12** (9), 880–886.

Sabaté, M., Prats, G., Moreno, E., Ballesté, E., Blanch, A. R. & Andreu, A. 2008 Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. *Res. Microbiol.* **159** (4), 288–293.

Santos, L. H., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barcelo, D. & Montenegro, M. C. 2013 Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: Identification of ecologically relevant pharmaceuticals. *Sci. Total Environ.* **461–462C**, 302–316.

Schubert, S., Picard, B., Gouriou, S., Heesemann, J. & Denamur, E. 2002 Yersinia high-pathogenicity island contributes to virulence in *Escherichia coli* causing extraintestinal infections. *Infect. Immun.* **70** (9), 5335–5337.

Tacão, M., Correia, A. & Henriques, I. 2012 Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *bla*(CTX-M)-like genes. *Appl. Environ. Microbiol.* **78** (12), 4134–4140.

Taylor, N. G., Verner-Jeffreys, D. W. & Baker-Austin, C. 2011 Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends Ecol. Evol.* **26** (6), 278–284.

Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. 1994 Genomic fingerprinting of bacteria using the repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* **5**, 25–40.

First received 22 May 2014; accepted in revised form 2 September 2014. Available online 24 September 2014