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Genetic characterization of fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital effluents

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ABSTRACT

Escherichia coli with reduced susceptibility to ciprofloxacin, isolated from urban streams, wastewater treatment plants and hospital effluent between 2004 and 2012, were compared based on multilocus sequence typing (MLST), quinolone and beta-lactam resistance determinants and plasmid replicon type. Isolates from the different types of water and isolation dates clustered together, suggesting the persistence and capacity to propagate across distinct aquatic environments. The most prevalent MLST groups were ST10 complex and ST131. Almost all isolates (98%) carried mutations in the chromosomal genes *gyrA* and/or *parC*, and 10% possessed the genes *qepA*, *aac(6')-Ib-cr* and/or *qnrS1*. Over 80% of the isolates were resistant to three or more classes of antibiotics (MDR ≥ 3). The most prevalent beta-lactamase encoding gene was *bla*TEM, followed by *bla*CTX-M-15, co-existing with plasmid mediated quinolone resistance. The plasmid replicon types of the group IncF were the most prevalent and distributed by different MLST groups. The genes *aac(6')-Ib-cr* and/or *qnrS1* could be transferred by conjugation in combination with the genes *bla*TEM,*bla*SHV-12 or *bla*OXA-1 and the plasmid replicon types I1-Iγ , K, HI2 and/or B/O. The potential of multidrug resistant *E. coli* with reduced susceptibility to ciprofloxacin, harboring mobile genetic elements and with ability to conjugate and transfer resistance genes, to spread and persist across different aquatic environments was demonstrated.

INTRODUCTION

The increase in the prevalence of antibiotic resistance has come into focus in the latter decades, frequently attributed to the continuous use of antibiotics and other antimicrobials (Martinez 2009; Tello, Austin and Telfer 2012). The occurrence of new types of resistance and new combinations of resistance genes is, in general, reported initially in clinical and/or veterinary settings (Acar 1997; Robicsek *et al.* 2006a; Kumarasamy *et al.* 2010) but detection in other environments, in particular in wastewater, is usually reported shortly after (Szczepanowski *et al.* 2009). Wastewater discharges are major sources of antibiotic resistant bacteria and resistance determinants, part of which will spread in the environment (Baquero, Martínez and Cantón 2008; Martinez 2009; Rizzo *et al.* 2013; Vaz-Moreira, Nunes and Manaia 2014). Although the fate of antibiotic resistance genes in the environment is not completely understood (Martı´nez and Baquero 2014), it has been demonstrated that some persist even in the absence of selective pressures (Andersson and Hughes 2012). Quinolones, antimicrobial therapeutic agents introduced in 1962, are a group of broad spectrum synthetic antibiotics used for a variety of infections. These drugs were expected to bypass the problem of resistant bacteria, since no resistance mechanism specific for this new class of antibiotics was supposed to exist in nature (Robicsek, Jacoby and Hooper 2006a). However, following quinolone introduction into the clinical practice, resistance to this class of antibiotics became commonly reported in diverse environments (Martínez- Martínez, Pascual and Jacoby 1998; Novo and Manaia 2010; Mokracka *et al.* 2011; Vredenburg *et al.* 2013). In *Enterobacteriaceae,* quinolone resistance emerged independently several times (Strahilevitz *et al.* 2009), and is associated with chromosome mutations and/or with plasmid-borne genes. The most frequent mechanism of resistance to quinolones in *Escherichia coli* includes mutations in the quinolone resistance-determining region of the genes *gyrA* and *parC* (Heisig 1996; Webber *et al.* 2013). Plasmid-associated quinolone resistance genes have also been described (Park *et al.* 2006; Cattoir *et al.* 2007; Pé richon, Courvalin and Galimand 2007; Cavaco *et al.* 2009; Wang *et al.* 2009). Al- though these genes confer only low-level resistance, it has been argued that their presence can favor the occurrence of chromosomal mutations, which will increase the minimal inhibitory concentration tolerated and, therefore, the resistance level (Robicsek *et al.* 2006b).

Conjugative plasmids are important elements for horizontal gene transfer, contributing to spread antibiotic resistance genes (Carattoli 2009; Parsley *et al.* 2010; Villa *et al.* 2010). For this rea- son, plasmids of ubiquitous and human-associated bacteria are regarded as interesting tools to track antibiotic resistance acquisition (Carattoli 2009). On the other hand, *E. coli*, a universal indicator of

fecal contamination due to its anthropogenic origin, is one of the most interesting bacterial groups to infer about the processes and paths of resistance dissemination. Members of this species harbor a myriad of plasmids, known to be associated with the capacity to acquire different families of resistance genes (Mammeri *et al.* 2005; Carattoli 2009; Shibl *et al.* 2012). Simultaneously, the emergence of virulent and multidrug resistant (MDR) *E. coli* strains (French 2010; Woodford, Turton and Livermore 2011) highlights the importance of monitoring antibiotic resistance determinants in environmental populations of these bacteria. *E. coli* sequence type (ST) 131 is of this a good example, since members of this group frequently combine virulence and multidrug resistance (Nicolas-Chanoine *et al.* 2008; Johnson *et al.* 2010; Rogers, Sidjabat and Paterson 2011).

In previous studies, we demonstrated that quinolone resistance was highly prevalent in Gram-negative bacteria (*E. coli* and *Aeromonas*) isolated from wastewater and that wastewater treatment could select for ciprofloxacin resistant *E. coli* (Ferreira da Silva *et al.* 2007; Figueira, Serra and Manaia 2011a; Figueira *et al.* 2011b). These results motivated a further research on the genetic characterization of fluoroquinolone resistant *E. coli* from waters with fecal contamination, such as urban streams, hospital effluents (HEs) and municipal wastewater, isolated over a period of nine years. In particular, we aimed to identify the genetic determinants of quinolone resistance in these isolates, the weight of vertical and horizontal transmission of this type of resistance and to assess if quinolone and beta-lactam resistance determinants in *E. coli* are transmitted simultaneously in the environment. Using multilocus sequence typing (MLST), plasmid replicon typing, antibiotic resistance phenotyping, quinolone and beta-lactam resistance genes detection and conjugation experiments, it was intended to assess: (a) the major quinolone resistance genes in these isolates and the associated beta-lactamase genes and plasmid replicons; (b) if specific MLST groups were associated with a given source, resistance profile or plasmid carriage; (c) if acquired quinolone resistance genes were harbored by conjugative plasmids.

METHODS

Bacterial isolates

A collection of 80 ciprofloxacin resistant *E. coli* isolates from raw wastewater (RWW, $n = 28$) and treated wastewater (TWW, $n = 33$) of four urban wastewater treatment plants, from urban streams and ponds (US, $n = 9$), herein designated as streams, and from untreated HE ($n = 10$) was analyzed (Fig. S1, Sup- porting Information). The collection comprised isolates recovered between 2004 and 2012, on antibiotic-free culture media ($n = 29$) or on the same media

supplemented with 4 mg L^{-1} ciprofloxacin ($n = 45$) or other antibiotics (16 mg L^{-1} tetracycline, $n = 1$; 32 mg L^{-1} amoxicillin, $n = 1$; 350 mg L^{-1} sulfamethoxazole, $n = 4$), as described in previous studies (Ferreira da Silva *et al.* 2007; Figueira, Serra and Manaia 2011a; Novo *et al.* 2013; Varela *et al.* 2014).

MLST analysis

The isolates were genotyped by MLST, based on the house- keeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* as proposed by Wirth *et al.* (2006). Allelic profiles and STs were determined based on *E. coli* MLST database queries (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Phylogenetic analysis was carried out using the concatenated sequences of the seven genes. Sequences were aligned using ClustalW and dendrograms were constructed using MEGA version 6 (Tamura *et al.* 2013). The evolutionary distances, estimated based on the model of Jukes and Cantor (1969). The dendrogram was constructed based on neighbor-joining method and its stability was con- firmed with the maximum likelihood method, using MEGA version 6 (Tamura, Nei and Kumar 2004, 2013).

Detection of plasmid replicons

The presence of plasmid replicons was determined as described by Carattoli *et al.* (2005), for FIA, FIB, FIC, HI1, HI2, I1-Iγ , L/M, N, P,W, T, A/C, K, B/O, X and Y, and by Villa *et al.* (2010) for the FII type. Positive controls were included in each PCR as- say and the authenticity of the test amplicons was confirmed based on nucleotide sequence analysis and query of public databases (http://www.ncbi.nlm.nih.gov/blast/). Isolates yielding replicons of the groups FIA, FIB and/or FII and plasmid mediated quinolone resistance or beta-lactamase genes (de- scribed below) were further characterized by plasmid MLST (http://pubmlst.org/plasmid/) (Villa *et al.* 2010).

Antibiotic resistance phenotypes

Antibiotic resistance phenotypes were determined based on the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012). Disks of amox- icillin (AML, 25 μg), ticarcillin (TIC, 75 μg), cephalothin (CP, 30 μg), ceftazidime (CAZ, 30 μg), meropenem (MEM, 10 μg), colistin (CT, 50 μg), sulfamethoxazole (SUL, 25 μg), sulfamethoxazole/trimethoprim (SXT, 25 μg), ciprofloxacin (CIP, 5 μg), tetra-cycline (TET, 30 μg), gentamycin (GEN, 10 μg) or streptomycin (STR, 10 μg) (Oxoid) were dispensed on Mueller-Hinton agar cultures, which incubated at 37ºC for 24 h. The MIC (minimum inhibitory concentration) of ciprofloxacin was

determined for selected isolates using strips embedded with an antibiotic gradient (MIC Evaluator, Oxoid). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 were included as quality control strains in all assays. Were considered MDR isolates exhibiting resistance to three or more different classes of antibiotics (penicillins, cephalosporins, carbapenems, amino-glycosides, quinolones, sulfonamides, tetracyclines, polymyx- ins) (Magiorakos *et al.* 2012).

Genetic determinants associated with ciprofloxacin or beta-lactam resistance

Chromosomal mutations in the genes *gyrA* and *parC* were analyzed in the nucleotide sequences of PCR amplicons generated with the primers gyrA6/gyrA631R and HJL3/HJL4, respectively, as described previously (Weigel *et al.* 1998; McDonald *et al.* 2001).

The detection of the genes *qnrA, qnrB, qnrC, qnrD, qnrS, qepA, qnrVC* and *aac(6')-Ib-cr*, encoding plasmid-mediated quinolone resistance, was performed as described by Figueira *et al.* (2011a,b) and Tacão *et al.* (2014). Isolates were also screened for the presence of the genes *oqx*AB, using the protocol described by Liu *et al.* (2011), with an annealing temperature of 55ºC. The following references were included in each PCR reaction and nucleotide sequence analysis as positive and quality control: *E. coli* L0 (*qnrA1*+), *Klebsiella pneumoniae* B1 (*qnrB1*+), *Enterobacter cloacae* S1 (*qnrS1*+) (Cattoir *et al.* 2007), *E. coli* TOP10+ paT851 (*qepA*+) (Périchon, Courvalin and Galimand 2007), *E. coli* DH10B transformant pHS11 (*qnrC*+) (Wang *et al.* 2009), *E. coli* DH10B transformant p2007057 (*qnrD*+) (Cavaco *et al.* 2009), *oqx*A+ and *oqx*B+ isolates from the laboratory's culture collection. The gene *aac(6')-Ib* was further inspected through nucleotide sequence analysis for detection of the mutations that characterize the *aac(6')-Ib-cr* variant (Robicsek *et al.* 2006b).

Beta-lactamase encoding genes commonly found in *E. coli* were also surveyed by PCR and nucleotide sequence determination. Primer sets and PCR conditions for *bla*OXA (Ouel- lette, Bissonnette and Roy 1987; Henriques *et al.* 2006), *bla*TEM and *bla*SHV (DiPersio *et al.* 2005) and *bla*CTX-M and *bla*OXY-2 (Weill *et al.* 2004; Monstein, Tärnberg and Nilsson 2009) were the same as reported in previous publications. The presence of beta-lactamase encoding genes from the *ampC* family (*bla*CMY and *bla*LAT) was also examined by PCR (Dierikx *et al.* 2010). Briefly, for these genes the following conditions were used for reactions of 25 μL: 1 μL DNA, 1 U of *Taq* DNA polymerase (ThermoScientific), 1 × KCl PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP's mix (ThermoScientific) and 20 pmol of each primer (5'-ATGATGAAAAAATCGTTATGCTGC-3' and 5'-

GCTTTTCAAGAATGCGCCAGG-3′), and a thermal cycling pro- gram consisting of 5 min at 94°C, 30 cycles of 1 min at 92°C, 1 min at 58°C and 1 min at 72°C and a final extension step of 10 min at 72°C (Biometra). Positive controls from the laboratory's culture collection were included in the PCR assays, and PCR product authenticity was confirmed based on nucleotide sequence analysis and database query, using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and the database at http://www.lahey.org /Studies/.

Conjugation assays

Isolates carrying plasmid-encoded quinolone resistance (PMQR) were further characterized for their ability to transfer resistance by conjugation, using the sodium azide resistant *E. coli* J53 as the recipient strain. Culture of donors and recipient strains grown in Luria-Bertani (LB) broth for 4 h were mixed in equal volumes, centrifuged at 10 000 rpm for 5 min, suspended in the same medium and incubated overnight at 28°C. Putative transconjugants were selected on LB agar plates supplemented with sodium azide (100 mg L^{-1}) and amoxicillin (32 mg L^{-1}) or ciprofloxacin (0.06 mg L^{-1}). Transconjugants were confirmed through random amplified polymorphic DNA genotyping and screened for the antibiotic susceptibility phenotypes, the presence of plasmid replicon types and resistance determinants detected in the donor strains, as described above.

Statistical analyses

The prevalence of antibiotic resistance and plasmid replicon type was compared among MLST groups and isolates recovered on ciprofloxacin-free/supplemented media, using Monte Carlo simulations of Fisher's exact test based on sampling 10 000 tables at a significance level (P) of <0.05. All statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Genotypic diversity

MLST analysis was used as a method to assess possible paths of transmission of fluoroquinolone resistant *E. coli* in waters with fecal contamination. From a collection of 776 *E. coli* isolates (Figueira, Serra and Manaia 2011a, Varela *et al*. 2014) recovered over a period of nine years from municipal wastewater, HE and urban streams 80 isolates with reduced susceptibility to ciprofloxacin were selected for further comparison. Most of the isolates yielded sequences matching 32 STs of the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The most commonly found types were ST10 complex (13 isolates) and ST131 (seven

isolates). The combination of the ST deter- mined for the seven alleles of five isolates did not match any entry in the database. These sequences were submitted as new STs, with the numbers (ST4460, ST4656, ST4659, ST4703 and ST4712).

Concatenated sequences corresponding to 3386 nucleotide positions were compared, leading to the establishment of five groups (A–E), formed at sequence identity values above 99% (more than 3352 identities). Three isolates that shared among them a similarity value of 98.6% formed an additional group, which given the small dimension was herein considered of un- clustered isolates. Groups A, B and D formed a major cluster (Fig. 1). Group A included 25 isolates, mostly from TWW but also from HE, RWW and urban streams. In group A, isolates were predominantly related with complex ST10, including ST10 and ST44. Group B was composed of eight isolates from urban wastewater treatment plants (TWW and RWW), related with the complexes ST469, ST46 and ST398. Group C included 10 isolates originated from all types of water sampled and mostly related with the ST131. Group D comprised 26 isolates also from all types of water and distributed by 18 ST. This group was the most genetically diverse and included bacteria related with the complexes ST155, ST156, ST205, ST40 and ST101. Group E was composed of eight isolates from urban wastewater and urban streams, related with the complex ST354. The three unclustered isolates were from RWW and TWW, and were related with the complexes ST31 and ST69. The frequency of isolates from ciprofloxacin-supplemented or antibiotic-free culture medium in each MLST group was non-significantly different ($P > 0.05$), suggesting that those isolation conditions did not influence the genetic diversity of the *E. coli* isolates recovered.

Plasmid replicon typing

Since plasmids are considered major drivers in processes of horizontal gene transfer, we were interested in characterizing the pattern of plasmid replicons in function of the phylogenetic diversity. It was possible to identify 12 plasmid replicon types out of the 18 that were screened for, suggesting a wide potential for propagation through horizontal gene transfer. The observed replicon types were randomly distributed by the different phylogenetic lineages and types of water (Fig. 1). The most prevalent replicon types were FII (53/80; 66.3%) and FIB (49/80; 61.3%), whereas all the others presented prevalence values below 26.5% (Table 1). The isolation conditions seemed to be related to the presence of the replicon type N ($n = 9$), which was only detected in isolates recovered on culture-medium with ciprofloxacin. By contrast, the presence of ciprofloxacin in the isolation culture medium was not associated with any significant differences

($P > 0.05$) in the prevalence of plasmid replicon types FIA, FIB, FII, I1-Iγ , K and B/O. In general, the most prevalent replicon types were randomly distributed by the different MLST groups. The only exceptions were the replicon type FIA, which was significantly ($P < 0.05$) more prevalent in group C (related with ST131) and the replicon type FII, significantly less prevalent ($P < 0.05$) in group D (related with ST155, ST156, ST297, ST443 and ST3024) (Table 1). Nucleotide sequences representative of each type of plasmid replicon found were deposited in the GenBank (Accession numbers LN714727 – 38).

Antibiotic resistance phenotypes

Resistance phenotypes were determined for antibiotics representative of eight classes of antibiotics. Most isolates were resistant to sulfamethoxazole (70/80), amoxicillin and ticarcillin (68/80), and tetracycline (62/80). The prevalence of resistance to ceftazidime, comparatively lower than resistance to other antibiotics ($n = 13/80$), was observed mainly in HE isolates ($P < 0.05$, 6/10) and absent in stream isolates. Although resistance phenotypes to meropenem and colistin were not observed, resistance to four or more classes of antibiotics (MDR4, including fluoroquinolones) was highly prevalent (82.1% in RWW, 81.8% in TWW, 80.0% in HE). In order to infer about the possible co-selection of resistance to fluoroquinolone and other antibiotics, the prevalence of resistance among isolates recovered on ciprofloxacin- supplemented ($n = 45$) and antibiotic-free culture medium ($n = 29$) was compared. The co-selection hypothesis was con- firmed for ceftazidime resistance, which was found to be significantly ($P < 0.05$) more prevalent among isolates recovered on ciprofloxacin supplemented than from antibiotic-free culture medium (11/45 versus 2/29). By contrast, the isolation of tetracycline resistant and multidrug resistant bacteria (MDR4) was significantly ($P < 0.05$) less frequent on ciprofloxacin-supplemented culture medium (17/45 and 13/32, respectively). When antibiotic resistance prevalence was compared among the five MLST groups (A–E), the only significant ($P < 0.05$) difference was the lower percentage of streptomycin resistance in group D (Table 1). Curiously, group D had also the lowest prevalence of replicon type FII.

Chromosomal determinants of quinolone resistance

Mutations in the chromosomal genes *gyrA* and/or *parC* could ex- plain the reduced ciprofloxacin susceptibility observed in 77 of the 80 isolates. A total of 76 isolates showed an amino acid ex- change in the position 83 of the DNA

gyrase, with the substitution of serine by leucine (TCG→TTG), either alone (n = 5) or combined with an additional mutation in the position 87. This second mutation consisted in the substitution of the aspartate by asparagine (GAC→ AAC, n = 70), glycine (GGC, n = 1) or tyrosine (TAC, n = 1) (Fig. 1). For a single isolate, this was the only mutation detected in *gyrA*. A total of 70 isolates also carried mutations in the gene *parC* (Fig. 1), the most common occurring in position 80 and consisting of the substitution of the serine by isoleucine (AGC →ATC, n = 54; AGC →ATT, n = 10), or less frequently by an arginine (AGC →AGA). In addition, two isolates presented a silent mutation in this position (AGC→AGT). Four isolates presented an additional mutation at position 84, in which glutamate was substituted by glycine (GGA, n = 3) or valine (GTA, n = 1). All isolates of group E presented the codon ATT for isoleucine instead of the more common ATC for in position 80. In two isolates, from RWW and TWW, neither *gyrA* nor *parC* yielded mutations associated with quinolone resistance. For six isolates, all clustered in group C and mostly related with the ST131, it was not possible to obtain pure sequences of the gene *par*C in spite of the multiple attempts made, suggesting a mutation in the area targeted by the primers. Curiously, all these isolates displayed MICCIP values higher than 32 μg mL^{-1} independently of the coupled *gyrA* mutations or presence of PMQR genes. It is worth of notice that isolates without mutations or carrying the gyrase mutations leucine (TTG) at position 83, tyrosine (TAC) or asparagine (AAC) at position 87, were recovered only from antibiotic-free culture medium, which indicates that these mutation profiles may be related to the respective MICCIP values of <4 μg mL^{-1} for the substitution Leu83Gly87 and >32 μg mL^{-1} for the substitution Leu83Tyr87.

Plasmid-encoded quinolone resistance

PMQR was detected in 10% (8/80) of the isolates from RWW and HE, but not in isolates from treated or urban streams. These genes were detected in isolates presenting quinolone resistance-conferring *gyrA* and/or *parC* mutations, and were only found in isolates from ciprofloxacin-supplemented culture medium. Most (7/8) isolates carrying PMQR displayed an elevated MICCIP of >32 μg mL^{-1}, although an identical MIC value could be observed in isolates that did not harbor any of the PMQR investigated (Fig. 1; Table 2). All eight isolates with PMQR were resistant to five or more classes of antibiotics (MDR5, n = 4; MDR6, n = 4) (Table 2). The gene a*ac(6')-Ib-cr* was the most common among the PMQR

determinants detected, and exclusively found in isolates from HE and RWW, related with ST93, ST744 and ST131. Two variants of this gene, with the transition TGG→CGG or the transversion TGG→AGG in the codon 102 were observed irrespective of the origin of the isolates. A single RWW isolate yielded the gene *aac(6')-Ib* without any of the three mutations conferring resistance to quinolones. The genes *qepA* and *qnrS1* were detected only in RWW isolates, with *qnrS1* co- occurring with *aac(6')-Ib-cr*. The gene *qepA* was detected in two isolates of the complex ST205 that in spite of high genotypic resemblance presented distinct resistance profiles and genetic determinants, evidencing processes of horizontal gene trans- fer. Both *qepA* gene sequences were identical, displaying neutral substitutions of phenylalanine (TTC) in position 95 for leucine (TTA), differing from *qepA1* (FJ744121.1) and *qepA2* (EU847537.1), and of valine (GTC) in position 134 for isoleucine (ATC), identical to *qepA1*. Novel *qepA* nucleotide sequences were deposited in the GenBank (Accession numbers LK934677-8). The genes *qnrA*, *qnrB*, *qnrC, qnrD, qnrVC* and *oqx*AB were not detected in any of the isolates.

All strains with PMQR presented plasmid replicons from the IncF group, as well as other replicon types such as I1-Iγ , B/O, K, HI2, N and A/C. According to the replicon MLST analysis, two isolates recovered from different sources in separate years (HE 2011, and RWW 2008) shared the allele combination FIA-; FIB1; FII24. Despite this similarity of plasmid replicon types, these isolates harbored different variants of the gene *aac(6')-Ib- cr,* and one (from RWW) carried also the gene *qnrS1,* suggesting possible recombination events (Table 2). Moreover, hinting at possible horizontal gene transfer, both isolates with identical plasmid replicons were genetically distinct, belonging to different clusters (A and D) and different lineages (ST744 and ST224, respectively).

Characterization of beta-lactamase-encoding genes

Since beta-lactam resistance was observed in 85% of the isolates and plasmid encoded beta-lactamases are often reported in quinolone resistant *E. coli*, genes related with beta-lactam resistance were also searched in these isolates. Genes encoding beta-lactamases were detected in 52 isolates. All isolates with PMQR presented at least one of the surveyed beta-lactamase encoding genes. The gene *bla*TEM was the most common, being present in isolates from all types of water and isolation conditions. Isolates carrying the gene *bla*CTX-M were all from urban wastewater and yielded MDR4 to MDR6 profiles. The vari- ant *bla*CTX-M-15 was predominant (7/8) and in two isolates of the ST131 and ST205 complex, and co-existed with the genes *aac(6')- Ib-cr,*

*bla*OXA-1, and *qepA*, respectively. The gene *bla*OXA-1 was ob- served in five isolates, distributed by all MLST clusters and types of wastewater, co-existing with the genes *bla*OXY-2, *bla*CTX-M-15, *bla*SHV or with PMQR genes (*qnrS1, aac(6')-Ib-cr*) (Fig. 1). The gene *bla*SHV-12 was only detected in HE ($n = 4$), in isolates distributed by different MLST groups, and, in one isolate, co-existed with the gene *aac(6')-Ib-cr*. The genes *bla*CTX-M-15, *bla*SHV-12 and *bla*OXA-1 were associated with the ceftazidime resistance phenotypes observed. Plasmid encoded beta-lactamase genes of the *ampC* family was not detected.

Conjugative transfer of PMQR or beta-lactamase genes

The eight isolates harboring PMQR were tested for their conjugation capacity with the strain *E. coli* J53 as recipient and se- lection of transconjugants on culture medium with 32 mg L^{-1} amoxicillin or 0.06 mg L^{-1} ciprofloxacin (Table 3). Selection on amoxicillin-supplemented culture medium allowed the recovery of five transconjugants and on ciprofloxacin of four. Of the six strains originating transconjugants, three were either re- covered on amoxicillin or on ciprofloxacin-supplemented cul- ture medium, and presented different characteristics for both selection conditions (Table 3). All amoxicillin transconjugants showed acquired phenotypes of resistance to amoxicillin, ticarcillin and sulfamethoxazole. Resistance acquired by transconjugants obtained on culture medium with ciprofloxacin varied between no acquired resistance and resistance to sulfamethoxazole, to amoxicillin/ticarcillin and to tetracycline. Plasmid replicon types acquired by conjugation comprised FIA, FII, I1-Iγ , K, B/O, HI2 and N, and among the beta-lactamase genes, *bla*TEM, *bla*SHV and *bla*OXA were transferred (Table 3). Transfer of PMQR was observed for three isolates from HE and RWW, selected on amoxicillin and ciprofloxacin. The gene *qnrS1* was transferred with the gene *bla*TEM and the replicon types K, B/O and I1-Iγ , and the gene *aac(6')-Ib-cr* with the genes *bla*OXA-1, *bla*SHV and the replicon type HI2. Some genetic elements could only be detected in transconjugants obtained in medium with ciprofloxacin (*bla*OXA, plasmid replicon B/O) or with amoxicillin (plasmid replicon I1-Iγ).

DISCUSSION

E. coli has been frequently investigated as an indicator of antibiotic resistance in the environment (e.g. Ferreira da Silva et al. 2007; Vredenburg *et al.* 2013; Amos *et al.* 2014). The combination of approaches which investigate the phylogenetic diversity of the bacterial hosts and simultaneously the genetic elements of resistance may bring new insights into several aspects related with

antibiotic resistance ecology. This was the basis for the design of this work, aiming at comparing *E. coli* isolates with reduced susceptibility to fluoroquinolones, recovered from water environments over a period of nine years. These strains, selected from a collection of 776 isolates were characterized in terms of genetic diversity and prevailing mechanisms of quinolone resistance, aiming to infer about the potential for PMQR horizontal gene transfer. Isolates from different origins, dates and conditions of isolation clustered together. These results suggest that the same *E. coli* lineages are dispersed over water environments as distinct as HE, urban wastewater treatment plant raw inflow and treated effluent and urban streams. Most of the observed MLST are ubiquitous, being frequently reported in association with humans (in clinical context or in the community) or in animal husbandry. STs prevalent in this study, such as ST10, ST131, ST167 and ST57, have been reported in clinical isolates (Coque *et al.* 2008; Oteo *et al.* 2009, 2010, 2012; Cao *et al.* 2011; Poulsen *et al.* 2013; Guillard *et al.* 2014), in wastewater impacted aquatic environments (Amos *et al.* 2014) and also in pets and livestock (Giufrè *et al.* 2012; Shepard *et al.* 2012; Mora *et al.* 2013; Pires-dos-Santos, Bisgaard and Christensen 2013; Schink *et al.* 2013; Wagner, Gally and Argyle 2014). Other STs detected in wastewater, such as ST533, ST648 and ST744 have been reported in pets (Huber *et al.* 2013; Schink *et al.* 2013; Wagner, Gally and Argyle 2014), and ST93, ST101, ST155, ST224 and ST1421 in live- stock (Hasan *et al.* 2012; Pires-dos-Santos, Bisgaard and Christensen 2013). This evidence suggests a possible circulation of the same lineages over different hosts and environmental compartments. The role of some of these lineages on antibiotic resistance propagation has been noted before, as clinical isolates are frequently associated to quinolone resistance (e.g. Jones *et al.* 2008) and extended-spectrum beta-lactamase genes (e.g. Mellmann *et al.* 2008; Valverde *et al.* 2009). In addition to antibiotic resistance, some of these lineages are also recognized by their virulence potential either in humans or livestock. Members of the ST131 and ST57, respectively, related with the phylogroups B2 and D, have been shown to carry a wide range of virulence genes associated with their MDR profiles (Johnson *et al.* 2010; Ben Sallem *et al.* 2012; Wagner, Gally and Argyle 2014). These evidences show that wastewater represents an important link in the circulation of bacteria of human and animal origin, al- though its role on the potential of transfer of these bacteria or resistance genes back to humans is still unclear.

In the present study, antibiotic resistance was found to be equally distributed among the different genetic lineages, suggesting the randomness of resistance acquisition. In contrast, the origin of isolates was determinant, with hospital and RWW representing major reservoirs of resistance, which suggests

that HE may have a role as a source of emerging resistance types. Resistance to ceftazidime, a third-generation cephalosporin, which according to recent reviews, is still not among the most prevalent resistant types in wastewater environments (Rizzo *et al.* 2013; Vaz-Moreira, Nunes and Manaia 2014) was mainly observed in isolates from HE that were recovered on ciprofloxacin-supplemented culture medium. The co- selection of quinolone and cephalosporin resistance phenotypes has been reported before for *Enterobacteriaceae* in clinical environment (Wener *et al.* 2010; Cao *et al.* 2011) and adds to the potential of bacteria to become MDR. In contrast, prevalence of tetracycline and MDR were lower among isolates from ciprofloxacin supplemented than from antibiotic-free culture medium. Al- though it may hint a bias imposed by the isolation procedure, this result suggests different paths of resistance acquisition for quinolones and tetracycline, as suggested in previous studies on the relationship between antibiotic residues and bacterial com-munity variations (Novo *et al.* 2013; Varela *et al.* 2014).

Quinolone resistance is transmitted by both vertical and horizontal transfer processes, although, at least in *E. coli*, vertical transmission is certainly of great importance (Uchida *et al.* 2010; Figueira, Serra and Manaia 2011a; Shibl *et al.* 2012; Vredenburg *et al.* 2013). The importance of vertical transmission was evidenced also in this study. A particularly interesting evidence was given by group E (n = 8) in which all isolates, recovered over a period of six years and from distinct types of water presented the same Ile80(ATT) mutation, different from all the others. How- ever, since chromosome mutations are apparently responsible for high MIC values, it is intriguing which kind of selective pressure may enhance horizontal gene transfer of PMQR or promote its selection. In this, as in previous studies, PMQR was preferentially found in bacteria isolated on ciprofloxacin-supplemented culture medium (Figueira *et al.* 2011b; Vredenburg *et al.* 2013). These observations suggest that PMQR may confer a selective advantage which is not necessarily related with an increased MIC value. Indeed, it was suggested that by conferring a low resistance level, PMQR may contribute to enhance the prevalence of resistant mutants in a population (Jacoby 2005). Accordingly, PMQR may act as facilitators for mutations acquisition, being lost in a subsequent stage, when they are no longer required. However, such an argument would not explain why PMQR was only detected among isolates from ciprofloxacin-supplemented culture medium. Moreover, the fact these determinants were only detected in raw effluents (from hospital and municipal treatment plants) may be an indication of a low stability in mobile genetic elements. On the other hand, while a certain instability may explain the low frequency observed, the association with distinct plasmid replicon types and

bacterial lineages, suggests a wide distribution and independent circulation of the determinants *aac(6')-Ib-cr* and *qnrS1*, also noted before (Park *et al.* 2006; Carattoli 2009; Guillard *et al.* 2014).

Through the analysis of distribution of PMQR over the distinct genetic lineages and associated plasmid replicons, it was expected to infer about the likelihood of horizontal gene transfer. The number of different replicon types was, in general, higher in isolates related with the STs ST10 complex and ST131, frequently reported in humans and animals and in clinical settings. Indeed, horizontal gene transfer are sup- posed to be favored in specific environments, preferentially directly subjected to anthropogenic impacts (Schlü ter *et al.* 2007; Martinez 2009).

The ability of MDR bacteria to transfer their resistance determinants to a recipient strain was demonstrated *in vitro*. Sup- porting the phylogenetic inferences, the ability to conjugate was not related with the origin of the isolate, genetic lineage or plasmid replicon type. Three of the eight isolates harboring PMQR were able to transfer the genes *aac(6')-Ib-cr* together with *bla*SHV and *bla*OXA (one isolate) or *qnrS1* together with *bla*TEM (two isolates) and the plasmid replicon types I1-Iγ , K, B/O and/or HI2. These plasmid replicon types have been found in the environment, particularly in wastewater (Moura *et al.* 2012; Tacão *et al.* 2014), but also in poultry (Dierikx *et al.* 2010) and clinical samples (Valverde *et al.* 2009; Oteo *et al.* 2012), showing their widespread dissemination and suggesting their high potential to spread antibiotic resistance through different environmental compartments. Curiously, replicon types on the group IncF were not observed to be associated with PMQR transfer.

It is concluded that *E. coli* strains with reduced susceptibility to quinolones obtained from HE, streams and wastewater are genetically closely related, suggesting that the same populations can move freely among these and other environmental compartments. The concern here is that clinically relevant populations discharged from hospital and municipal effluents can reach the environment and persist in aquatic environments. Reduced susceptibility to quinolones was observed to be transferred through both vertical and horizontal gene transfer, mainly the genes *aac(6')-Ib-cr* and *qnrS1* both demonstrated to be located on conjugative plasmids.

The results presented herein suggest that clinically relevant strains with complex antibiotic-resistant profiles that develop under the selective pressure of the hospital settings can be disseminated to the environment through wastewater discharges. Preventive measures, like separation of human excretions suspected of carrying emerging and/or high doses of

antibiotic resistance and treatment of HE, could delay the global spread of such bacteria and their genetic determinants.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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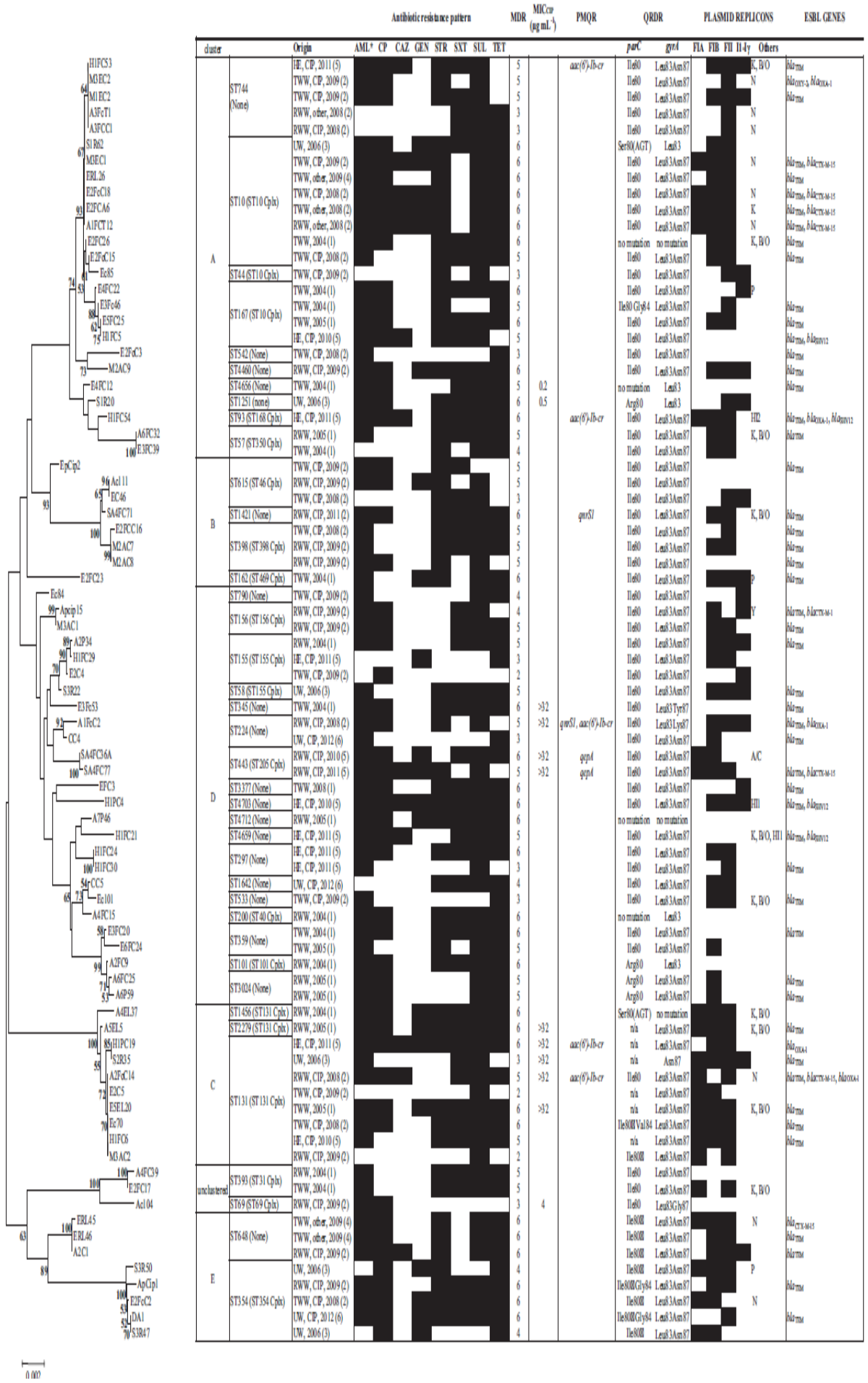
Antibiotic resistance pattern
MDR
MIC$_{CIP}$ (μg mL^{-1})
PMQR
QRDR
PLASMID REPLICONS
ESBL GENES
cluster
Origin
AML* CP CAZ GEN STR SXT SUL TET
parC
gyrA
FIA FIB FII I1-γ Others
A
B
C
D
E
unclustered
ST744 (None)
ST10 (ST10 Cplx)
ST44 (ST10 Cplx)
ST167 (ST10 Cplx)
ST542 (None)
ST4460 (None)
ST4656 (None)
ST1251 (none)
ST93 (ST168 Cplx)
ST57 (ST350 Cplx)
ST615 (ST46 Cplx)
ST1421 (None)
ST398 (ST398 Cplx)
ST162 (ST469 Cplx)
ST790 (None)
ST156 (ST156 Cplx)
ST155 (ST155 Cplx)
ST58 (ST155 Cplx)
ST345 (None)
ST224 (None)
ST443 (ST205 Cplx)
ST3377 (None)
ST4703 (None)
ST4712 (None)
ST4659 (None)
ST297 (None)
ST1642 (None)
ST533 (None)
ST200 (ST40 Cplx)
ST359 (None)
ST101 (ST101 Cplx)
ST3024 (None)
ST1456 (ST131 Cplx)
ST2279 (ST131 Cplx)
ST131 (ST131 Cplx)
ST393 (ST31 Cplx)
ST69 (ST69 Cplx)
ST648 (None)
ST354 (ST354 Cplx)
H1FCS3
M3EC2
M1EC2
A3FcT1
A3FCC1
SI R62
M3EC1
ERL26
E2FcC18
E2FCA6
A1FCT12
E2FC26
E2FcC15
Ec85
E4FC22
E3Fc46
E5FC25
H1FC5
E2FcC3
M2AC9
E4FC12
S1R20
H1FC54
A6FC32
E3FC39
EpCip2
Acl 11
EC46
SA4FC71
E2FCC16
M2AC7
M2AC8
E2FC23
Ec84
Apcip15
M3AC1
A2P34
H1FC29
E2C4
S3R22
E3Fc53
A1FcC2
CC4
SA4FC36A
SA4FC77
EFC3
H1PC4
A7P46
H1FC21
H1FC24
H1FC30
CC5
Ec101
A4FC15
E3FC20
E6FC24
A2FC9
A6FC25
A6P59
A4EL37
A5EL5
H1PC19
S2R3 5
A2FcC14
E2C5
E5EL20
Ec70
H1FC6
M3AC2
A4FC39
E2FC17
Acl 04
ERL45
ERL46
A2C1
S3R50
ApCip1
E2FcC2
DA1
S3R47
0.009

Figure 1. Neighbor-joining dendrogram based on the concatenated nucleotide sequences of the housekeeping genes *adk*, *fumC, gyrB, icd, mdh, purA* and *recA* (number of positions = 3386) of *E. coli* strains with reduced susceptibility to quinolones. Bootstrap values greater than 50% based on 1000 replications are given at the internal nodes. Black dots indicate branches recovered also based on the maximum likelihood method. Origin: HE, HE, RWW and TWW, urban wastewater treatment plant raw inflow and TWW, respectively, US, urban streams; CIP, recovered in media supplemented with 4 mg L^{-1} ciprofloxacin; other, recovered in media supplemented with other antibiotics (32 mg L^{-1} amoxicillin, 16 mg L^{-1} tetracycline or 350 mg L^{-1} sulfamethoxazole); AML, amoxicillin; TIC, ticarcillin; CP, cephalothin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. MDR, number of classes of antibiotics to which resistance was detected. *Resistance to AML and TIC was similar for all isolates. All isolates were susceptible to meropenem and colistin. n/a, no sequence available. Ser80(AGT), silent mutation; a, AGC → ATT mutation. (1) Ferreira da Silva *et al.* (2007); (2) Novo and Manaia (2010); (3) Figueira *et al.* (2011a), (4) Varela *et al.* (2014);

(5) this study.

Table 1. Prevalence of antibiotic resistance and/or plasmid-carriage in isolates of each MLST group.

MLST groups	Plasmid replicons (%)								Antibiotic resistance (%)									
	FIA ($n = 21$)	FIB ($n = 49$)	FII ($n = 53$)	N ($n = 9$)	I1-Iγ ($n = 18$)	K ($n = 11$)	B/O ($n = 10$)	Other[b]	AML ($n = 68$)	TIC ($n = 68$)	CP ($n = 55$)	CAZ ($n = 13$)	GEN ($n = 21$)	STR ($n = 57$)	SXT ($n = 48$)	SUL ($n = 70$)	TET ($n = 62$)	MDR4 ($n = 66$)
A ($n = 25$)	20.0	60.0	84.0	24.0	24.0	16.0	12.0	P, HI2	84.0	84.0	72.0	28.0	24.0	84.0	60.0	92.0	80.0	84.0
B ($n = 8$)	0.0	37.5	62.5	0.0	25.0	12.5	12.5	P	87.5	87.5	50.0	0.0	25.0	100.0	62.5	87.5	50.0	87.5
C ($n = 10$)	88.9[a]	100.0	100.0	11.1	11.1	33.3	33.3	–	80.0	80.0	60.0	20.0	40.0	60.0	80.0	100.0	70.0	70.0
D ($n = 26$)	7.7	61.5	42.3[a]	0.0	30.8	7.7	7.7	Y, HI1, A/C	88.5	88.5	69.2	11.5	19.2	50.0[a]	57.7	84.6	80.8	76.9
E ($n = 8$)	50.0	87.5	75.0	25.0	12.5	0.0	0.0	P	75.0	75.0	100.0	12.5	50.0	87.5	37.5	75.0	100.0	100.0
other ($n = 3$)	33.3	0.0	33.3	0.0	0.0	33.3	33.3	–	100.0	100.0	33.3	0.0	0.0	66.7	66.7	66.7	66.7	66.7
Total ($n = 80$)	26.3	61.3	66.3	11.3	22.5	13.8	12.5		85.0	85.0	68.8	16.3	26.3	71.3	60.0	87.5	77.5	81.3

AML, amoxicillin; TIC, ticarcillin; CP, cephalotin, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. All isolates were susceptible to meropenem and colistin; MDR4, resistant to 4 or more classes of antibiotics; a, significantly different ($p < 0.05$) from the other groups; b, less than 3% of the plasmid replicons detected.

Table 2. Genetic characteristics of isolates harboring plasmid mediated quinolone resistance genes.

Origin	Group	ST (ST Cplx)	*parC*	*gyrA*	FAB formula other plasmid replicons	Plasmid resistance genes	*aac(6′)-Ib-cr* mutations	Antibiotic resistance pattern	MDR	MIC_{CIP} ($\mu g\ mL^{-1}$)	Isolate
HE	A	ST744 (None)	Ile80	Leu83Asn87	F24:A-:B1 I1-Iγ, K, B/O	*aac(6′)-Ib-cr* bla_{TEM}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/ STR/SXT, SUL	5	8	H1FC53
HE	A	ST93 (ST168 Cplx)	Ile80	Leu83Asn87	F18:A1:B1 HI2	*aac(6′)-Ib-cr* bla_{TEM} bla_{OXA-1} bla_{SHV-12}	102:AGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/ STR/SXT, SUL/TET	6	>32	H1FC54
HE	C	ST131 (ST131 Cplx)	n/a	Leu83Asn87	F31:A-:B1 P	*aac(6′)-Ib-cr* bla_{OXA-1}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/GEN, STR/SXT, SUL/TET	6	>32	H1PC19
RWW	B	ST1421 (None)	Ile80	Leu83Asn87	F18:A-:B8 K, B/O	*qnrS1* bla_{TEM}	–	AML, TIC/CP/STR/SXT, SUL/TET	6	>32	SA4FC71
RWW	C	ST131 (ST131 Cplx)	Ile80	Leu83Asn87	F40:A1:B-N	*aac(6′)-Ib-cr* bla_{TEM} $bla_{CTX-M-15}$ bla_{OXA-1}	102:CGG,117:TTA,179:TAT	AML, TIC/CP, CAZ/SXT, SUL/TET	5	>32	A2FCC14
RWW	D	ST224 (None)	Ile80	Leu83Asn87	F24:A-:B1 I1-Iγ	*qnrS1*, *aac(6′)-Ib-cr* bla_{TEM} bla_{OXA-1}	102:AGG,117:TTA,179:TAT	AML, TIC/CP/STR/SUL	5	>32	A1FCC2
RWW	D	ST443 (ST205 Cplx)	Ile80	Leu83Asn87	F-:A1:B26 A/C	*qepA*	–	AML, TIC/CP/GEN/SXT, SUL/TET	6	>32	SA4FC36A
RWW	D	ST443 (ST205 Cplx)	Ile80	Leu83Asn87	F1:A1:B1	*qepA* bla_{TEM} $bla_{CTX-M-15}$	–	AML, TIC/CP, CAZ/GEN, STR/SUL	5	>32	SA4FC77

All isolates were susceptible to meropenem and colistin; MDR, number of classes of antibiotics to which a resistance phenotype was found; n/a, no sequence available. HE – hospital effluent, RWW and TWW – raw inflow and treated wastewater from wastewater treatment plants.

Table 3. Characteristics of transconjugants from isolates harboring plasmid mediated quinolone resistance genes selected on amoxicillin or ciprofloxacin.

				Antibiotic resistance pattern											
Isolate Transconjugant	Selection on	origin	ST/ST cmplx	AML/ TIC	CP	CAZ	GEN	STR	SXT	SUL	TET	MIC_{CIP}* ($\mu g\ mL^{-1}$)	Plasmid replicons	PMQR	Betalactamases
H1FC53		HE	ST744 (None)	R	R	R	S	R	R	R	S	8	FIB, FII, I1/Iγ, K, B/O	*aac(6′)-Ib-cr*	bla_{TEM}
T_{AML}.h1fc53	AML			R	R	S	S	R	S	R	S	0.015	I1/Iγ, K	nd	bla_{TEM}
H1FC54		HE	ST93 (ST168 Cplx)	R	R	R	S	R	R	R	R	>32	FIA, FIB, FII, HI2	*aac(6′)-Ib-cr*	bla_{OXA-1}, bla_{SHV}, bla_{TEM}
T_{AML}.h1fc54	AML			R	R	S	S	S	S	R	S	0.25	HI2	*aac(6′)-Ib-cr*	bla_{SHV}
T_{CIP}.h1fc54	CIP			R	R	S	S	S	S	R	R	0.18	HI2	*aac(6′)-Ib-cr*	bla_{SHV}, bla_{OXA-1}
H1PC19		HE	ST131 (ST131 Cplx)	R	R	R	R	R	R	R	R	>32	FIB, FII	*aac(6′)-Ib-cr*	bla_{OXA-1}
T_{CIP}.h1pc19	CIP			S	S	S	S	S	S	S	S	12	nd	nd	nd
A1FcC2		RWW	ST224 (None)	R	R	S	S	R	S	R	S	>32	FIB, FII, I1/Iγ	*qnrS*, *aac(6′)-Ib-cr*	bla_{OXA-1}, bla_{TEM}
T_{AML}.a1fcc2	AML			R	S	S	S	S	S	R	S	0.03	I1/Iγ	*qnrS*	bla_{TEM}
T_{CIP}.a1fcc2	CIP			R	S	S	S	S	S	S	S	0.12	nd	*qnrS*	bla_{TEM}
A2FcC14		RWW	ST131 (ST131 Cplx)	R	R	R	S	S	R	R	R	>32	FIA, FII, N	*aac(6′)-Ib-cr*	bla_{CTX-M}, bla_{OXA-1}, bla_{TEM}
T_{AML}.a2fcc14	AML			R	S	S	S	S	R	R	R	0.015	FIA, FII, N	nd	bla_{TEM}
SA4FC71		RWW	ST1421 (None)	R	R	S	S	R	R	R	R	>32	FIB, FII, K, B/O	*qnrS*	bla_{TEM}
T_{AML}.sa4fc71	AML			R	S	S	S	S	S	R	S	0.25	K	*qnrS*	bla_{TEM}
T_{CIP}.sa4fc71	CIP			R	S	S	S	S	S	S	S	0.12	K, B/O	*qnrS*	bla_{TEM}

AML, amoxicillin; TIC, ticarcillin; CP, cephalotim, CAZ, ceftazidime; GEN, gentamycin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; TET, tetracycline. All isolates were susceptible to meropenem and colistin; MDR, number of classes of antibiotics to which resistance was displayed. *donor strain *E.coli* J53 $MIC_{CIP} = 0.015\ \mu g\ mL^{-1}$. nd – not detected.