Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Genotypic characteristics of multidrug-resistant *Pseudomonas aeruginosa* from hospital wastewater treatment plant in Rio de Janeiro, Brazil

C.C. Miranda[1], I. de Filippis[1], L.H. Pinto[2], T. Coelho-Souza[1], K. Bianco[1], L.C. Cacci[3], R.C. Picão[3] and M.M. Clementino[1]

1 Instituto Nacional de Controle de Qualidade em Saúde, FIOCRUZ, Rio de Janeiro, Brazil
2 Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil
3 Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Keywords
clonal complexes, hospital sewage, multidrug-resistant, multilocus sequence typing, *Pseudomonas aeruginosa*.

Correspondence
Maysa M. Clementino, Fundação Oswaldo Cruz, Instituto Nacional de Controle de Qualidade em Saúde. Avenida Brasil, 4.365 CEP: 21040-900, Rio de Janeiro-RJ, Brazil.
E-mail: maysa.mandetta@incqs.fiocruz.br

2014/2387: received 20 November 2014, revised 16 January 2015 and accepted 24 February 2015

doi:10.1111/jam.12792

Abstract

Aims: To investigate *Pseudomonas aeruginosa* isolates from a hospital wastewater treatment plant (HWTP), focusing on enzyme-based mechanisms of β-lactams resistance and the genetic relatedness among isolates.

Methods and Results: Forty-one *Ps. aeruginosa* strains recovered from a HWTP were identified by amplification of 16S rRNA gene. β-lactamase production was screened by disc diffusion, CHROMagar extended-spectrum β-lactamase (ESBL) and β-lactamase strips. β-lactamase and ESBL producing isolates were investigated by PCR for the presence of ESBL, metallo-β-lactamase and *Klebsiella pneumoniae* carbapenemase encoding genes. Thirty-four isolates (83%) were resistant to at least one antibiotic belonging to three or more classes. Out of these 34 isolates, 28 (82%) were classified as multidrug-resistant (MDR) and 6 (18%) extensively drug-resistant (XDR). Genetic relatedness by Enterobacterial Repetitive Intergenic Consensus sequence-PCR and Multilocus sequence typing analysis showed 20 distinct profiles and 15 sequencing types respectively. Clonal Complex 244 (CC244) shows the pathogenic potential of this clone carrying MDR and XDR strains from clinical, environmental and hospital waste sources.

Conclusions: Our results suggest that treatment facilities for hospital wastewater can stimulate the increase of antimicrobial resistance bacteria and genes.

Significance and Impact of the Study: The great genetic diversity of *Ps. aeruginosa* recovered from HWTP constantly released into aquatic systems allow the spread of antimicrobial-resistant organisms and genes.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen with intrinsic resistance to many antimicrobials. Furthermore, under selective pressure, this micro-organism may easily develop powerful resistance either by mutation in chromosomally encoded genes or by horizontal transfer of resistance genes (Zhao and Hu 2010). Infections caused by this pathogen are often difficult to treat because of its multidrug-resistant (MDR) phenotype (De Francesco *et al.* 2013).

Production of β-lactamases, such as extended-spectrum β-lactamases (ESBLs) and carbapenemases, is an important mechanism of β-lactam antibiotics resistance in *Ps. aeruginosa* nosocomial isolates, which jeopardizes antimicrobial therapy in hospitalized patients (Picão *et al.* 2009). Carbapenemase production is of particular concern as it confers resistance to all β-lactams including extended-spectrum cephalosporins, monobactams and carbapenems (Zavascki *et al.* 2010). Carbapenemases that were identified in *Ps. aeruginosa* so far include: metallo-β-lactamases (MBLs) of the VIM (Verona imipenemase),

IMP (imipenemase), SPM (São Paulo metallo β-lactamase), GIM (German imipenemase) AIM (Australian imipenemase), DIM (Dutch imipenemase) and NDM types (New Delhi metallo-β-lactamase); KPC (*Klebsiella pneumoniae* carbapenemase), GES (Guiana extended spectrum) types (GES-2, -4, -5, -6 and -11) (Poirel *et al.* 2002; Gupta 2008; Jovcic *et al.* 2011) and OXA (Oxacillinase) type carbapenemases (Poirel *et al.* 2007, 2010). Among these resistance mechanisms, SPM-1, VIM-2, IMP-1, IMP-16, GES-5 and KPC-2 (Toleman *et al.* 2002; Mendes *et al.* 2004; Sader *et al.* 2005; Martins *et al.* 2007; Picão *et al.* 2009; Silva *et al.* 2011; Jácome *et al.* 2012) were found in *Ps. aeruginosa* from Brazil, most of which were clinical isolates.

Hospital effluents may represent a great risk to human public health due to the presence of pathogens and chemicals such as disinfectants, anaesthetics, heavy metals, antimicrobial agents and other drugs that are not metabolized by patients (Emmanuel *et al.* 2005). The disposal of this liquid waste in the treatment of hospital wastewater treatment plant (HWTP) can favour, the selective pressure, an increase in bacterial populations with phenotypes of multidrug resistance to antimicrobials (Prado *et al.* 2008).

Many carbapenem-resistant *Ps. aeruginosa* isolates and clinically important ESBLs-and carbapenemase-encoding genes have been found in hospital wastewater (Santoro *et al.* 2012), hospital and municipal sewage (Korzeniewska *et al.* 2013), urban sewerage systems (containing rainwater, hospital and urban wastewater) (Slekovec *et al.* 2012) wastewater contaminated rivers (Fontes *et al.* 2011; Amos *et al.* 2014). Of notice, NDM-1-producing *Ps. aeruginosa* was identified in tap water collected in India (Walsh *et al.* 2011).

In order to circumvent this problem, hospital effluents have been subjected to different treatments. However, several studies have showed that antibiotic resistance bacteria and genes persist in effluents of a variety of full-scale managements at levels above those typical of aquatic environments, even after disinfection (Auerbach *et al.* 2007).

The aim of this study was to investigate the antimicrobial susceptibility profiles and genetic relatedness of *Ps. aeruginosa* isolates obtained from a HWTP. We also explored the β-lactam-encoding genes and the clonal relationships among the isolates using Multilocus sequence typing (MLST) scheme.

Materials and methods

Study setting and sewage sampling

The effluent treatment system studied is located at a healthcare complex in the metropolitan area of Rio de Janeiro city, Brazil. The facility holds 322 beds and receives around 30 000 persons per month. The hospital contains a wastewater treatment plant that treats 220 cubic meters of sewage per day. It performs tertiary treatment in four stages, including pretreatment to remove gross solids; an aeration tank (continuous stirred tank reactor with sludge recycle), a clarifier tank and a post-treatment (disinfection of final effluent by chlorination). The treated effluent is discharged in rainwater network and then, into water bodies such as rivers and seawater.

Samples were collected in 2008 and in 2010 from five steps across the station (Grid-affluent, aeration tank, sludge, chlorination tank and treated effluent). Five hundred millilitres per site were collected approximately 20 cm below the surface in sterile glass bottles. Samples were kept refrigerated (4°C) until processed in the laboratory, within 8 h of collection. Temperature, pH, conductivity, dissolved oxygen, turbidity and salinity was assessed at the time of sample collection using Water Quality Checker U-10 (HORIBA, Kyoto, Japan) and dosage of chlorine was carried out using chlorine meter (Homis, São Paulo, Brazil).

Isolation and identification of *Pseudomonas aeruginosa*

A 100 ml-aliquot of each sample was filtered on membranes of 0·45 μm porosity. The membranes were transferred to selective asparagine broth and were incubated at 37°C for 24 h. The appearance of green fluorescence under ultraviolet light was considered positive for *Ps. aeruginosa* growth. Aliquots of 1 ml from these tubes were transferred to acetamide broth and were incubated at 37°C for 24 h. Tubes in which the development of pink colour was observed had an aliquot streaked on acetamide broth (Fuentefria *et al.* 2011). After incubation at 37°C for 24 h, six colonies per plate were selected for further studies, comprising a total of 60 isolates. Isolates were identified phenotypically using Vitek 2 GNI cards following the manufacturer's recommendations (bioMerieux, Marcy L'etoile, France) and results were certified by *Ps. aeruginosa*-specific PCR analysis (Spilker *et al.* 2004).

Antimicrobial susceptibility and MDR/XDR classification

The isolates were tested for antibiotic susceptibility by disc diffusion according to the Clinical Laboratory Standard Institute (CLSI 2014). *Escherichia coli* (ATCC 25922) and *Ps. aeruginosa* (ATCC 27853) were used as quality control strains. Seventeen antimicrobial agents were tested: amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg) gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), levofloxacin (5 μg),

piperacillin/tazobactam (100/10 μg), ticarcillin/clavulanic acid (75/10 μg), tobramycin (10 μg), polymyxin B (300 units), colistin (10 μg) and fosfomycin (200 μg).

The isolates were classified as MDR and extensively drug-resistant (XDR) according to Magiorakos *et al.* (2012). Production of β-lactamases and ESBLs was investigated by β-lactamase strip (Probac, São Paulo, Brazil) and CHROMagar—ESBL (CHROMagar, Paris, France) respectively. Statistical significance ($P < 0{\cdot}05$) between resistance profiles of each step were inferred by Fisher or chi-square test.

Detection of ESBL, MBL and KPC genes

The β-lactamase and ESBL positive isolates were screened by PCR for the presence of ESBL (bla_{PER}, bla_{VEB}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}25}$, bla_{TEM} and bla_{GES}), MBL (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM}) and KPC (bla_{KPC}) encoding genes, as previously described (Toleman *et al.* 2002; Nagano *et al.* 2004; Dubois *et al.* 2005; Poirel *et al.* 2011; Doyle *et al.* 2012; Monteiro *et al.* 2012; Wang *et al.* 2012; Ahmed *et al.* 2013; Barguigua *et al.* 2013).

ERIC typing analysis

Genotypic analysis of the strains was investigated by amplification of the Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR). The extraction of genomic DNA was performed using the protocol for Gram-negative Dnaeasy®Blood&Tissuet (Qiagen®, Valencia, CA, USA) according to the manufacturer's instructions and the primer ERIC2 were used for the amplification as previously described (Versalovic *et al.* 1991). The amplicons were analysed by gel electrophoresis and stained with ethidium bromide (3 mg ml^{-1}). The gel was photographed and analysed using ImageQuant300 (GE, Oppsala, Sweden). Band patterns were analysed with BIONUMERICS ver. 6.6 (Applied Maths, Kortrijk, Belgium) using the Dice coefficient and unweighted pair group method with arithmetic mean pair group method with arithmetic average. Isolates with 100% level of similarity were considered clonally related.

Multilocus sequence typing

The ancestral relationship of the *Ps. aeruginosa* isolates were analysed by MLST with a representative strain of each ERIC type. Most primers used for amplification and sequencing of seven housekeeping genes (*acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E) were designed (L. Cacci, personal communication) with primer-blast assistance (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with exception of *acs*A-F (Curran *et al.* 2004). The amplification of the housekeeping genes was performed as previously described and the nucleotide sequences were sequenced with fluorescent terminators (BigDye; Applied Biosystems, Foster City, CA) on an Applied Biosystems ABI Prism 3730 automated DNA sequences in duplicate for each primer (Otto *et al.* 2008).

Nucleotide sequences were submitted to the MLST database to determine the allelic numbers and Sequence Types (ST). Association of related ST to form clonal complexes (CCs) based on the number of identical alleles shared by ST is not a well-defined rule and can be subjective. The *Ps. aeruginosa* MLST database describes only two CCs: PA01 associated with ST-549 and PA14 associated with ST-253 and no information is disclosed of how these CCs were formed as there is only one ST for each CC. Maâtallah *et al.* (2013) considered that ST sharing five or more identical alleles are part of the same CC.

The MLST profiles were clustered with the BIONUMERICS 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) using a categorical coefficient and graphing was assessed using the minimum spanning tree tool, as described before (Schouls *et al.* 2004).

Results

Physical and chemical parameters

The pH of the five steps of the first sample analysed was maintained between 6·0–6·8, while in the second sample between 7·6 and 8·4, thus within the range of pH that enables the growth of many micro-organisms, including *Ps. aeruginosa*. Turbidity showed high values in step 1 and step 2 both samplings, mainly due to the presence of suspended solids in the water. The dissolved oxygen levels had the highest in step 2 where aeration occurs and elevates the levels of Dissolved Oxygen (DO), which is essential for organic matter biodegradation. The temperature of the five steps of two samplings remained at 28–30°C favouring mesophilic forms present. The chlorine concentration was high enough at the step 4 which the liquid chlorine is added to the tank. Mainly due to the presence of hypochlorite ion in high concentration, the conductivity was also high at this point that is directly proportional to the ionization of substances dissolved in the liquid (Table 1).

Pseudomonas aeruginosa identification and antimicrobial susceptibility profile

The five steps of sewage treatment at the HWTP yielded 60 isolates, 41 of which were identified as *Ps. aeruginosa*: step 1 – Grid/affluent ($n = 6$); step 2 – aeration tank ($n = 9$); step 3 – sludge ($n = 10$); step 4 – chlorination tank ($n = 4$); step 5 – treated effluent ($n = 12$).

Disc-diffusion susceptibility testing indicated high resistance to various antimicrobial agents. Highest resistance rate was observed for fosfomycin (88%) followed by ticarcillin/clavulanic acid (71%), ceftriaxone (63%), aztreonam (59%), cefotaxime (54%), imipenem (39%), cefepime (24%), meropenem (22%), ceftazidime (20%), tobramycin (20%), polymyxin B (20%), colistin (17%), gentamicin (15%), ciprofloxacin (12%), levofloxacin (12%), piperacillin/tazobactam (12%) and amikacin 2%). Susceptibility levels to fosfomycin, tobramycin, levofloxacin, ciprofloxacin and piperacillin/tazobactam were lower for the first three steps of effluent treatment (grid, aeration and sludge), with significant increase ($P < 0{\cdot}05$) on the fourth and fifth steps (chlorination and treated effluent).

Thirty-four out of 41 *Ps. aeruginosa* (83%) presented multidrug resistance profiles and were classified as MDR (82%) and XDR (18%) distributed along the five-step points of the HWTP. Thirty-eight isolates (93%) produced β-lactamase enzymes with 29 (76·3%) ESBL (Fig. 1).

ESBL, KPC and MBL-encoding genes

Among 29 (71%) isolates exhibiting ESBL phenotype, 13 harboured bla_{TEM} (45%), 7 harboured bla_{SHV} (24%) and 1 harboured $bla_{\text{CTX-M-1}}$ (3·5%). It is important to emphasize, however, that we did not sequence the amplicons obtained, thus temoniera (TEM) and sulfhydryl variable (SHV) production may not necessarily confer the ESBL phenotype observed. Thirty-eight (93%) isolates showed antimicrobial resistance for carbapenems, 14 carried bla_{KPC} (37%), 14 (37%) presented bla_{VIM} and 6 presented bla_{SPM} (18%) (Table 2).

ERIC and MLST genotyping

ERIC-PCR was able to fingerprint and assign a specific profile to all *Ps. aeruginosa* strains studied from different steps from a HWTP. Among all studied strains, ERIC-PCR resolved 20 genotypes. The banding patterns were highly reproducible under visual and automated analysis (Fig. 2).

MLST analysis revealed a total of 15 different ST, with five previously described (ST-238, ST-244, ST-381, ST-595 and ST-1621) and 10 new ST which were deposited in the *Ps. aeruginosa* MLST database (ST-1853, ST-1854, ST-1855, ST-1856, ST-1857, ST-1858, ST-1859, ST-1860, ST-1861, ST-1862) (Table 3). Discriminatory power of ERIC-PCR was higher than MLST as strains belonging to the same ST showed different ERIC profiles. Non-MDR (resistant less than three antibiotics) isolates belonged to single ST (238 and 381), with the exception of ST-244, ST-1621 and ST-1862. ST-244 was present in four of the five steps of HWTP, while the new ST-1859 was present in two steps. These two ST were represented by the larger number of isolates for each ($n = 8$) (Fig. 3). The 41 isolates were grouped into 15 different ST, with 10 newly described. Among the new ST, nine were associated with at least one MDR strain and one strain belonging to ST-1854 showed a XDR pattern. Other XDR strains were classified as ST-244 and ST-595 and were deposited in the *Ps. aeruginosa* MLST database.

Discussion

We investigated *Ps. aeruginosa* isolates from the HWPT and determined their antibiotic resistance profile, focusing in particular on enzyme-based mechanisms of resistance to β-lactams and in the determination of the genetic relatedness among isolates. Our results may suggest that wastewater treatment is followed by an increase in resistance profiles, although a quantitative study should be conducted to confirm this hypothesis. Nevertheless, it should be emphasized that Chagas *et al.* (2011) reported 48 and 21% of resistance to cefotaxime and ciprofloxacin, respectively, in Enterobacteriaceae isolates from hospital wastewater. Similar findings regarding *E. coli* from hospital wastewater being resistant to quinolones and cephalosporins have been

Table 1 Physical and chemical parameters of HWTP steps

	Abiotic parameters													
	pH		Conductivity $\mu S\ cm^{-1}$		Turbidity NTU		DO mg l^{-1}		Temperature °C		Salinity %		Chlorine ppm	
Site (step)	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010
Grid (1)	6·8	7·6	0·39	0·82	18	10	4·4	4·5	29	28	0·0	0·0	1	0·0
Aeration (2)	6·1	7·9	0·31	0·38	26	99	7·6	9·2	30	29	0·0	0·0	1	−1
Sludge (3)	6·1	8·2	0·29	0·38	14	6	1·5	9·3	30	29	0·0	0·0	1	−1
Chlorination (4)	6·3	8·4	0·52	0·48	12	4	3·5	9·2	30	29	0·0	0·0	10	10·9
Treated Effluent (5)	6·0	8·4	0·29	0·35	13	7	3·4	3·7	30	29	0·0	0·0	1	0·01

$\mu S\ cm^{-1}$, micro-Siemens per centimetre; NTU, Nephelometric Turbidity Unit; mg l^{-1} Milligrams per litre; °C, Celsius degrees; %, Per cent; ppm, Parts per million; HWTP, hospital wastewater treatment plant.

Hospital wastewater treatment plant
Primary treatment
Secondary treatment
Tertiary treatment
Grid
Aeration
Sludge
Chlorination
Treated effluent
Rainwater Network
Step 1
Step 2
Step 3
Step 4
Step 5
STRAIN
FOSFO
TIM
CTR
AZT
CTX
IMI
CPM
MEM
CAZ
TOB
POL
COL
GEN
CIP
LEV
PTZ
AMI
Profile

Figure 1 Susceptibility of isolates of the five steps of hospital wastewater treatment plant. Filled square, antimicrobial resistance; empty square, antimicrobial susceptibility; β, Beta-lactamase producers; *, extended-spectrum β-lactamase producers; M, multidrug-resistance; X, extensively drug-resistant.

reported among others from Denmark (Jakobsen *et al.* 2008), China (Han *et al.* 2012) and Polland (Korzeniewska and Harnisz 2013). Thus, HWTPs could be a hot spot for antibiotic-resistant bacteria selection (Pruden *et al.* 2013).

Table 2 β-lactamase genes detected in all the isolates of *Pseudomonas aeruginosa*

Isolates	β-lactamase genes	Isolation step
P3229	bla_{SHV}, bla_{KPC}	1
P3233	–	2
P3234	bla_{TEM}	3
P3238	bla_{TEM}, bla_{SPM}, bla_{VIM}	4
P3239	bla_{TEM}, bla_{VIM}	4
P3240	bla_{TEM}, bla_{KPC}, bla_{VIM}	5
P3241	–	5
P3242	–	5
P3246	bla_{TEM}	5
P3249	bla_{SHV}, bla_{VIM}	2
P3252	bla_{SHV}, $bla_{KPC,}$ bla_{VIM}	4
P3671	–	1
P3672	bla_{SPM}	1
P3673	bla_{SPM}	1
P3674	$bla_{SHV,}$ bla_{SPM}	1
P3675	bla_{VIM}	1
P3676	bla_{TEM}	2
P3677	bla_{KPC}, bla_{VIM}	2
P3678	bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$,$bla_{KPC}$	2
P3679	bla_{KPC}	2
P3680	bla_{KPC}	2
P3681	bla_{KPC}	2
P3682	bla_{KPC}	3
P3683	–	3
P3684	–	3
P3685	–	3
P3686	bla_{TEM}	3
P3687	bla_{KPC}	3
P3688	bla_{KPC}	3
P3689	bla_{TEM}, bla_{KPC}	3
P3691	bla_{TEM}	5
P3692	bla_{TEM}, bla_{VIM}	5
P3693	bla_{VIM}	5
P3694	$bla_{VIM,}$ bla_{SPM}	5
P3695	$bla_{VIM,}$ bla_{SPM}	5
P3696	–	5
P3697	–	5
P3846	bla_{TEM}, bla_{SHV}, $bla_{KPC,}$ bla_{VIM}	2
P3847	$bla_{KPC,}$ bla_{VIM}	3
P3848	bla_{TEM}, bla_{VIM}	4
P3849	bla_{TEM}	5

Isolation steps: (1) Grid/affluent; (2) aeration tank; (3) Sludge; (4) Chlorination tank; (5) Treated effluent.

Pseudomonas aeruginosa strains were recovered from all five stages of the HWTP studied, indicating that this species could be considered a good marker for studies aimed at evaluating the impact of wastewater treatment process on the prevalence of antibiotic resistance organisms and genes in the effluent. All stages of the station showed isolates exhibiting MDR pattern, in contrast, Slekovec *et al.* (2012), did not find resistant neither MDR isolates in HWTP before its release to the environment and in the sludge produced by the waste plant in France.

It is noteworthy to emphasize that 100% of the isolates of the chlorination step showed XDR profile. Using chemical disinfection to inactivate pathogens also plays an important role in controlling antibiotic-resistant bacteria in HWTPs. Studies on the effect of chlorination on antibiotic-resistant bacteria can be traced back to 1970s, where chlorination was shown to influence the proportion of multiple-antibiotic-resistant bacteria in drinking water and wastewater (Grabow *et al.* 1973; Armstrong *et al.* 1982; Murray *et al.* 1984). On the other hand, in a previous study, we demonstrated the absence of microbial growth in the chlorination step. However, *Ps. aeruginosa* cells detected in the following stage of the treatment revealed the capacity of regrowth in chlorinated sewage effluent maybe due to the bacteriostatic effect of chlorine, which justifies the presence of viable cells in stage 5 (Santoro *et al.* 2012).

Among strains exhibiting β-lactamase encoding genes, bla_{TEM}, bla_{SHV} $bla_{CTX\text{-}M\text{-}1}$, $bla_{KPC,}$ bla_{VIM} and bla_{SPM} were characterized randomly in 78% of the isolates. It is necessary to highlight that isolates carring bla_{TEM}, $bla_{KPC,}$ bla_{VIM} and bla_{SPM} were found in the fifth and final stage of the hospital wastewater with potential to spread throughout the aquatic environment, thus enabling human exposure and transmission.

Quintera *et al.* (2005) reported the isolation of a *Pseudomonas pseudoalcaligenes* VIM-2 strain from hospital sewage. This discovery suggests that the ongoing spread of the $bla_{VIM\text{-}2}$ is occurring simultaneously in several dimensions, as it can now be found in different environments and in several bacterial species. Another study revealed the presence of $bla_{VIM\text{-}2}$ in two unrelated *Ps. aeruginosa* isolates from aquatic environments (Quintera and Peixe 2006). The $bla_{SPM\text{-}1MBL}$ gene is the most prevalent in Brazil; its presence was evaluated in *Ps. aeruginosa* isolates from hospital sewage and surface-water samples, in order to obtain epidemiological data on the dissemination of this gene in environmental samples in southern Brazil (Fuentefria *et al.* 2009). The presence of

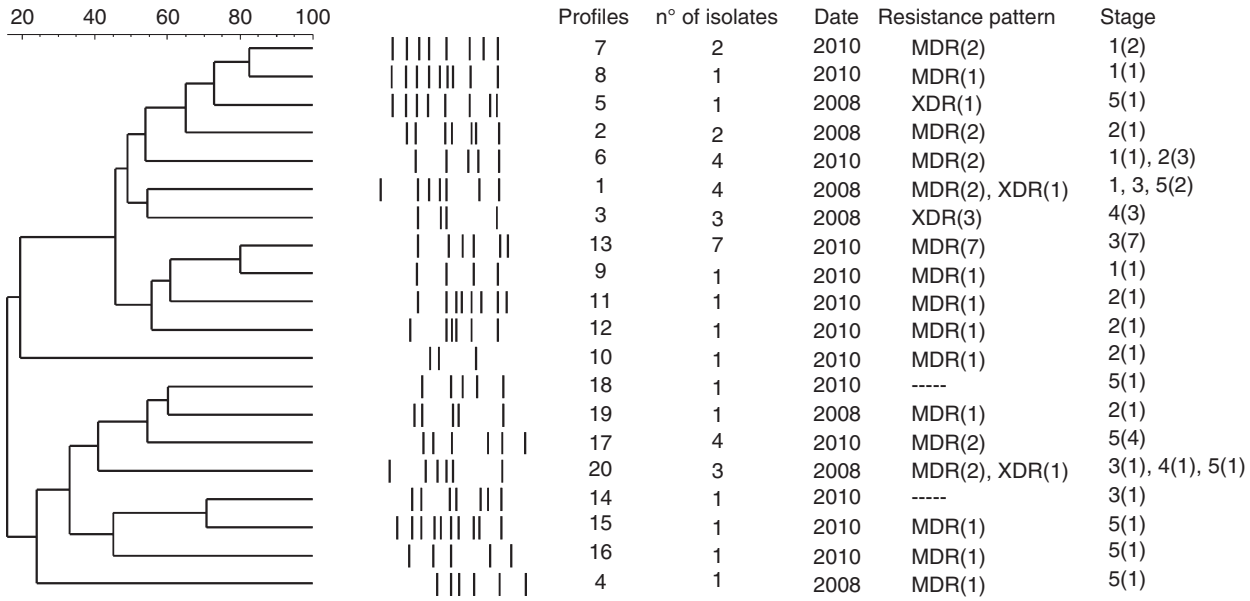

Figure 2 Cluster analysis by enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprint (ERIC 2) of 41 *Pseudomonas aeruginosa* isolates. Clustering analysis was performed with aid of BIONUMERICS 6.6 (Applied Maths) and based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean.

Table 3 MLST of isolates representing 20 *Pseudomonas aeruginosa* distinct profiles from hospital wastewater treatment plants during 18-month period

				Allelic profile						
ERIC profile	No of isolates	Collected step	ST	*acs*A	*aro*E	*gua*A	*mut*L	*nuo*D	*pps*A	*trp*E
1	4	1, 3, 5	244	17	5	12	3	14	4	7
2	2	2	1853	16	22	11	5	4	4	10
3	3	4	244	17	5	12	3	14	4	7
4	1	5	244	17	5	12	3	14	4	7
5	1	5	1854	17	5	44	110	14	81	7
6	4	1, 2	1621	5	54	99	48	1	6	3
7	2	1	1855	5	54	44	48	14	81	3
8	1	1	1856	30	2	44	110	1	97	7
9	1	1	1857	28	54	44	110	4	79	3
10	1	2	1858	17	2	11	3	81	38	3
11	1	2	1859	17	5	36	7	27	4	5
12	1	2	1858	17	2	11	3	81	38	3
13	7	3	1859	17	5	36	7	27	4	5
14	1	3	381	11	20	1	65	4	4	10
15	1	5	1860	17	5	44	3	14	77	5
16	1	5	1861	17	20	49	110	11	79	10
17	4	5	1862	17	20	12	110	4	81	10
18	1	5	238	5	1	59	6	1	33	42
19	1	2	1853	16	22	11	5	4	4	10
20	3	3, 4, 5	595	17	5	12	5	14	4	7

ERIC, enterobacterial repetitive intergenic consensus; MLST, multilocus sequence typing; ST, sequence types.

pathogenic Enterobacteriaceae from a hospital effluent showed resistance to third-generation cephalosporins, cefepime, aminoglycosides, quinolones and a significant rate of carbapenem. Genes encoding ESBL (bla_{SHV}, bla_{TEM}, bla_{CTX-M} and bla_{GES}) were detected among 46 strains with reduced susceptibility to third-generation cephalosporins isolated from hospital sewage (Pitout *et al.* 2004; Coque *et al.* 2008; Naas *et al.* 2008).

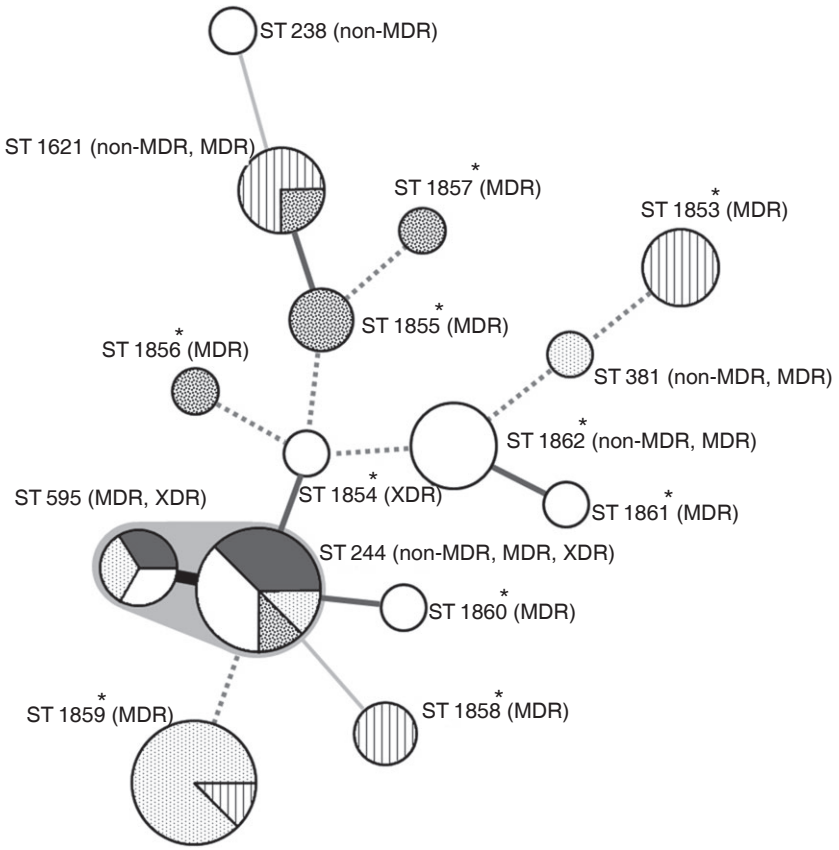

Figure 3 Minimum spanning tree of MLST sequence types of *Ps. aeruginosa* isolates from hospital wastewater treatment plant (HWTP). The tree was based on the analysis of concatenated sequences of acsA, aroE, guaA, mutL, nuoD, ppsA and trpE genes. Multidrug-resistant and extended drug-resistant strains are indicated beside each sequence type (ST). Circles indicate specific ST and the number of strains according to the size of the circle. Circle patterns indicate the HWTP step. New ST are marked with (*). Lines connecting ST groups, indicate that they differ in one allele (thick solid line), or three to five locus (thin and dotted lines). The halo surrounding ST-244 and ST-595 indicates a clonal complex (CC244). Relations between ST were determined by BioNumerics analysis (BIONUMERICS 6.6) where CC were formed by ST with single or double locus variation. (▩) Grid; (▥) Aeration; (▦) Sludge; (■) Chlorination and (□) Treated effluent.

The release of high concentrations of antibiotics and resistance genes in natural ecosystems is a recent event in evolutionary terms. However, these pollution can impact the structure and the activity of environmental microbial populations. Given that environmental micro-organisms are the original source of resistance genes acquired by human pathogens (Davies 1997). In water bodies, bacteria from different origins (human, animal, environmental) are able to mix, and resistance evolves as a consequence of promiscuous exchange and shuffling of genes and genetic vectors (Baquero *et al.* 2008).

The discharge of MDR bacteria including ESBL, KPC and MBL producers into an urban river is worrisome, as these isolates could persist in the environment and act as opportunistic pathogens and/or resistance reservoirs that could accelerate the evolution of antimicrobial resistance in the community (Kim and Aga 2007; Baquero *et al.* 2008; Martinez 2009).

The MLST analysis showed a high diversity, as reported in other previous studies (Gomila *et al.* 2013). ST244 is well distributed worldwide with 34 isolates in the MLST database being four isolated in Brazil (three from this study) and one clinical sample (Maâtallah *et al.* 2013; Bae *et al.* 2014; Chen *et al.* 2014;). Two strains belonging to ST595 deposited in the MLST database were isolated in Brazil both from nonclinical samples (one from this study). It is noteworthy to point out that the chlorination step showed the greatest number of isolates of MDR and XDR strains belonging to CC244 where all the isolates from this step were grouped (Fig. 3). To our knowledge, this is the first MLST analysis of *Ps. aeruginosa* isolated from hospital effluent.

Following the rule of Maâtallah *et al.* (2013), 11 CC, sharing five or more identical alleles, were described with two major clones formed by five ST each. One of these CC (CC244) named by its central ST-244, included other closely related ST (ST-990, ST-993, ST-986 and ST-654). Two ST belonging to CC244 (ST-244 and ST-654), included several clinical strains showing reduced susceptibility against different classes of antibiotics such as ESBL, IMP-type metallo-beta-lactamase and KPC producing. We could not compare strains associated with ST-990, ST-986 and ST-654 also included in CC244, as these ST were deleted from the MLST database and no data associated with these strains was available.

The three strains classified as ST-595 were beta-lactamase producers and showed reduced susceptibility with one strain showing a KPC and metallo-beta-lactamase phenotype (MDR), one ESBL and metallo-beta-lactamase (XDR) and one ESBL producer (MDR). Other eight

strains classified as ST-244 and also included in CC244 showed similar susceptibility patterns with several MDR and XDR strains KPC, ESBL and metallo-beta-lactamase producers. To date, CC244 has been detected among strains isolated from different sources such as clinical and environmental (Cholley *et al.* 2014). We first report, to our knowledge, the presence of strains belonging to CC244 from hospital effluent. These data suggest a possible turnover of strains sharing different antibiotic resistance mechanisms regardless of environment location.

The presence of MDR and XDR strains into CC244 suggests the pathogenic potential of this clone carrying strains with reduced susceptibility to several antimicrobial classes spreading through different sources from clinical to hospital effluent and the environment.

The spreading of MDR organisms and genes through the environment is worrying. Strategies to reduce this picture are required, including neutralization of antibiotics in wastewater and in the environment (Zwiener and Frimmel 2000; Ternes *et al.* 2003; Berglund *et al.* 2014).

Our results demonstrated the presence of isolates carrying important resistance mechanisms with potential to contaminate aquatic environments with increasing antibiotic resistance within the microbial community, leading to negative impacts on the environment and human health.

Acknowledgements

The authors are grateful to the Plataforma Genomica-Sequenciamento de DNA-RPT01A-PDTIS/FIOCRUZ. We acknowledge the financial support from CNPq (474204/2012-0), FAPERJ (E-26/112.657/2012) and INCQS-FIOCRUZ.

Conflict of Interest

No conflict of interest declared.

References

Ahmed, O.I., EL-Hady, S.A., Ahmed, T.M. and Ahmed, I.Z. (2013) Detection of bla_{SHV} and bla_{CTX-M} genes in ESBL producing *Klebsiella pneumoniae* isolated from Egyptian patients with suspected nosocomial infections. *Egypt J Med Hum Genet* **14**, 277–283.

Amos, G.C., Hawkey, P.M., Gazem, W.H. and Wellington, E.M. (2014) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. *J Antimicrob Chemother* **69**, 1785–1791.

Armstrong, J.L., Calomiris, J.J. and Seidler, R.J. (1982) Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Appl Environ Microbiol* **44**, 308–316.

Auerbach, E.A., Seyfried, E.E. and McMahon, K.D. (2007) Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res* **41**, 1143–1151.

Bae, I.K., Suh, B., Jeong, S.H., Wang, K.K., Kim, Y.R., Yong, D. and Lee, K. (2014) Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Korea producing β-lactamases with extended-spectrum activity. *Diagn Microbiol Infect Dis* **79**, 373–377.

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

Barguigua, A., El Otmani, F., Talmi, M., Reguig, A., Jamali, L., Zerouali, K. and Timinouni, M. (2013) Prevalence and genotypic analysis of plasmid-mediated β-lactamases amongurinary *Klebsiella pneumoniae* isolates in Moroccan community. *J Antibiot* **66**, 11–16.

Berglund, B., Khan, G.A., Weisner, S.E., Ehde, P.M., Fick, J. and Lindgren, P.E. (2014) Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. *Sci Total Environ* **1**, 476–477.

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

Chen, Y., Sun, M., Wang, M., Lu, Y. and Yan, Z. (2014) Dissemination of IMP-6-producing *Pseudomonas aeruginosa* ST244 in multiple cities in China. *Eur J Clin Microbiol Infect Dis* **33**, 1181–1187.

Cholley, P., Ka, R., Guyeux, C., Thouverez, M., Guessennd, N., Ghebremedhin, B., Frank, T., Bertrand, X. *et al.* (2014) Population structure of clinical *Pseudomonas aeruginosa* from West and Central African countries. *PLoS ONE* **9**(9), e107008.

Clinical and Laboratory Standards Institute (2014) *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement*. CLSI document M100–S23. Wayne, PA: CLSI.

Coque, T.M., Baquero, F. and Canton, R. (2008) Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveill* **13**, n.47.

Curran, B., Jonas, D., Grundmann, H., Pitt, T. and Dowson, C.G. (2004) Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J Clin Microbiol* **42**, 5644–5649.

Davies, J.E. (1997) Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found. Symp.* **207**, 15–27.

De Francesco, M.A., Ravizzola, G., Peroni, L., Bonfanti, C. and Manca, N. (2013) Prevalence of multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in an Italian hospital. *J Infect Public Health* **6**, 179–185.

Doyle, D., Peirano, G., Lascols, C., Lloyd, T., Church, D.L. and Pitout, J.D. (2012) Laboratory detection of Enterobacteriaceae that produce carbapenemases. *J Clin Microbiol* **50**, 3877–3880.

Dubois, V., Arpin, C., Noury, P., Andre, C., Coulange, L. and Quentin, C. (2005) Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. *J Clin Microbiol* **43**, 4129–4138.

Emmanuel, E., Perrodin, Y., Keck, G., Blanchard, J.M. and Vermande, P. (2005) Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. *J Hazardous Mat* **117**, 1–11.

Fontes, L.C., Neves, P.R., Oliveira, S., Silva, K.C., Hachich, E.M., Sato, M.I. and Lincopan, N. (2011) Isolation of *Pseudomonas aeruginosa* coproducing metallo-β-lactamase SPM-1 and 16S rRNA methylase RmtD1 in an urban river. *Antimicrob Agents Chemother* **55**, 3063–3064.

Fuentefria, D.B., Ferreira, A.E., Gräf, T. and Corção, G. (2009) Spread of Metallo-β-lactamases: screening reveals the presence of a *blaSPM-1* gene in hospital sewage in southern Brazil. *Braz J Microbiol* **40**, 82–85.

Fuentefria, A.D.B., Ferreira, A.E. and Corção, G. (2011) Antibiotic-resistant *Pseudomonas aeruginosa* from hospital wastewater and superficial water: are they genetically related? *J Environ Manage* **92**, 250–255.

Gomila, M., Del Carmen Gallegos, M., Fernández-Baca, V., Pareja, A., Pascual, M., íaz-Antolín, P., García-Valdés, E. and Lalucat, J. (2013) Genetic diversity of clinical *Pseudomonas aeruginosa* isolates in a public hospital in Spain. *BMC Microbiol* **13**, 138.

Grabow, W.O.K., Middendorff, I.G. and Prozeskv, O.W. (1973) Survival in maturation ponds of coliform bacteria with transferable drug resistance. *Water Res* **7**, 1589–1597.

Gupta, V. (2008) Metallo beta lactamases in *Pseudomonas aeruginosa* and *Acinetobacter* species. *Expert Opin Investig Drugs* **17**, 131–143.

Han, N., Sheng, D. and Xu, H. (2012) Role of *Escherichia coli* strain subgroups, integrons, and integron-associated gene cassettes in dissemination of antimicrobial resistance in aquatic environments of Jinan, China. *Water Sci Technol* **66**, 2385–2392.

Jácome, P.R., Alves, L.R., Cabral, A.B., Lopes, A.C. and Maciel, M.A. (2012) First report of KPC-producing *Pseudomonas aeruginosa* in Brazil. *Antimicrob Agents Chemother* **56**, 4990.

Jakobsen, L., Sandvang, D., Hansen, L.H., Bagger-Skjot, L., Westh, H., Jorgensen, C., Hansen, D.S., Pedersen, B.M. *et al.* (2008) Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the wastewater environment. *Environ Int* **34**, 108–115.

Jovcic, B., Lepsanovic, Z., Suljagic, V., Rackov, G., Begovic, J., Topisirovic, L. and Kojic, M. (2011) Emergence of NDM-1 metallo-β-lactamase in *Pseudomonas aeruginosa* clinical isolates from Serbia. *Antimicrob Agents Chemother* **55**, 3929–3931.

Kim, S. and Aga, D.S. (2007) Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health B Crit Rev* **10**, 559–573.

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

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

Maâtallah, M., Bakhrouf, A., Habeeb, M.A., Turlej-Rogacka, A., Iversen, A., Pourcel, C., Sioud, O. and Giske, C.G. (2013) Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*: comparison of their congruence with multi-locus sequence typing. *PLoS ONE* **8**, e82069.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F. *et al.* (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**, 268–281.

Martinez, J.L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* **157**, 2893–2902.

Martins, A.F., Zavascki, A.P., Gaspareto, P.B. and Barth, A.L. (2007) Dissemination of *Pseudomonas aeruginosa* producing SPM-1-like and IMP-1-like metallo-β-lactamases in hospitals from southern Brazil. *Infection* **35**, 457–460.

Mendes, R.E., Toleman, M.A., Ribeiro, J., Sader, H.S., Jones, R.N. and Walsh, T.R. (2004) Integron carrying a novel metallo-β-lactamase gene, blaIMP-16, and a fused form of aminoglycoside-resistant gene aac(6')-30/aac(6')-Ib': report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob Agents Chemother* **48**, 4693–4702.

Monteiro, J., Widen, R.H., Pignatari, A.C., Kubasek, C. and Silbert, S. (2012) Rapid detection of carbapenemase genes by multiplex real-time PCR. *J Antimicrob Chemother* **67**, 906–909.

Murray, G.E., Tobin, R.S., Junkins, B. and Kushner, D.J. (1984) Effect of chlorination on antibiotic resistance pro files of sewage-related bacteria. *Appl Environ Microbiol* **48**, 73–77.

Naas, T., Poirel, L. and Nordmann, P. (2008) Minor extended-spectrum beta-lactamases. *Clin Microbiol Infect* **14**, 42–52.

Nagano, N., Nagano, Y., Cordevant, C., Shibata, N. and Arakawa, Y. (2004) Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. *J Clin Microbiol* **42**, 3978–3984.

Otto, T.D., Vasconcellos, E.A., Gomes, L.H.F., Moreira, A.S., Degrave, W.M., Mendonça-Lima, L. and Alves-Ferreira,

M. (2008) ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. *Genet Mol Res* **7**, 861–871.

Picão, R.C., Poirel, L., Gales, L.C. and Nordmann, P. (2009) Diversity of β-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. *Antimicrob Agents Chemother* **53**, 3908–3913.

Pitout, J.D., Hossain, A. and Hanson, N.D. (2004) Phenotypic and molecular detection of CTX-M-beta-lactamases produced by *Escherichia coli* and *Klebsiella* spp. *J Clin Microbiol* **42**, 5715–5721.

Poirel, L., Weldhagen, G.F., De Champs, C. and Nordmann, P. (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J Antimicrob Chemother* **9**, 561–565.

Poirel, L., Pitout, J.D. and Nordmann, P. (2007) Carbapenemases: molecular diversity and clinical consequences. *Future Microbiol* **2**, 501–512.

Poirel, L., Naas, T. and Nordmann, P. (2010) Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrob Agents Chemother* **54**, 24–38.

Poirel, L., Revathi, G., Bernabeu, S. and Nordmann, P. (2011) Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. *Antimicrob Agents Chemother* **55**, 934–936.

Prado, T., Pereira, W.C., Silva, D.M., Seki, L.M., Carvalho, A.P. and Asensi, M.D. (2008) Detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Lett Appl Microbiol* **46**, 136–141.

Pruden, A., Larsson, D.G., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S. *et al.* (2013) Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ Health Perspect* **121**, 878–885.

Quintera, S. and Peixe, L. (2006) Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* **72**, 3743–3745.

Quintera, S., Ferreira, H. and Peixe, L. (2005) First isolation of *bla*VIM-2 in an environmental isolate of *Pseudomonas pseudoalcaligenes*. *Antimicrobial Agents Chemother* **49**, 2140–2141.

Sader, H.S., Reis, A.O., Silbert, S. and Gales, A.C. (2005) IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. *Clin Microbiol Infect* **11**, 73–76.

Santoro, D.O., Romão, C.M. and Clementino, M.M. (2012) Decreased aztreonam susceptibility among *Pseudomonas aeruginosa* isolates from hospital effluent treatment system and clinical samples. *Int J Environ Health Res* **22**, 560–570.

Schouls, L.M., van der Heide, H.G., Vauterin, L., Vauterin, P. and Mooi, F.R. (2004) Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. *J Bacteriol* **186**, 5496–5505.

Silva, F.M., Carmo, M.S., Silbert, S. and Gales, A.C. (2011) SPM-1-producing *Pseudomonas aeruginosa*: analysis of the ancestor relationship using multilocus sequence typing, pulsed-field gel electrophoresis, and automated ribotyping. *Microb Drug Resist* **17**, 215–220.

Slekovec, C., Plantin, J., Cholley, P., Thouverez, M., Talon, D., Bertrand, X. and Hocquet, D. (2012) Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. *PLoS ONE* **7**, e49300.

Spilker, T., Coenye, T., Vandamme, E.P. and Lipuma, J.J. (2004) PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas species* recovered from cystic fibrosis patients. *J Clin Microbiol* **42**, 2074–2079.

Ternes, T.A., Stüber, J., Herrmann, N., McDowell, D., Ried, A., Kampmann, M. and Teiser, B. (2003) Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Res* **37**, 1976–1982.

Toleman, M.A., Simm, A.M., Murphy, T.A., Gales, A.C., Biedenbach, D.J., Jones, R.N. and Walsh, T.R. (2002) Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY Antimicrobial Surveillance Program. *J Antimicrob Chemother* **50**, 673–679.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacterial and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

Walsh, T.R., Weeks, J., Livermore, D.M. and Toleman, M.A. (2011) Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* **11**, 355–362.

Wang, X.R., Chen, J.C., Kang, Y., Jiang, N., An, S.C. and Gao, Z.C. (2012) Prevalence and characterization of plasmid-mediated blaESBL with their genetic environment in *Escherichia coli* and *Klebsiella pneumoniae* in patients with pneumonia. *Chin Med J* **125**, 894–900.

Zavascki, A.P., Carvalhaes, C.G., Picão, R.C. and Gales, A.C. (2010) Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. *Expert Rev Anti Infect Ther* **8**, 71–93.

Zhao, W.H. and Hu, Z.Q. (2010) Beta-lactamases identified in clinical isolates of *Pseudomonas aeruginosa*. *Crit Rev Microbiol* **36**, 245–258.

Zwiener, C. and Frimmel, F.H. (2000) Oxidative treatment of pharmaceuticals in water. *Water Res* **34**, 1881–1885.