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FULL PAPERS

Occurrence of bacteria producing broad-spectrum beta-lactamases and *qnr* genes in hospital and urban wastewater samples

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SUMMARY

The aims were to investigate the level of antibiotic-resistant bacteria in hospital and urban wastewater and to determine the similarity of isolates obtained from wastewater and hospitalized patients.
Wastewater samples were collected in September 2013 and 2014.
After identification using MALDI-TOF MS, beta-lactamase production was determined by relevant phenotypic tests. Genes responsible for the production of single beta-lactamase groups and Qnr proteins were established. The epidemiological relationship of the isolates from wastewater and hospitalized patients was determined by PFGE.
A total of 51 isolates of enterobacteria were obtained.
Overall, 45.1% of them produced broad-spectrum beta-lactamases. Genes encoding TEM, SHV, CTX-M, CIT, DHA and EBC types of enzymes and Qnr proteins were detected. No broad-spectrum beta-lactamase production was confirmed in the urban wastewater treatment plant. The most important finding was the detection of two identical isolates of *K. pneumoniae* in 2013, one from a patient's urinary catheter and the other from a wastewater sample.

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INTRODUCTION

Bacterial resistance to antibiotics currently represents a very serious and complex problem transcending the borders of healthcare. According to the European Antimicrobial Resistance Surveillance Network (EARS-Net) interactive database (European Centre for Disease Prevention and Control 2015), an alarming occurrence of some resistance patterns is evident throughout the European countries. Examples of such resistance phenotypes are strains of *Klebsiella pneumoniae* and *Escherichia coli* resistant to 3rd generation cephalosporins (in the Czech Republic, 13.1% for *E. coli* and 52% for *K. pneumoniae* respectively; especially through the production of broad-spectrum beta-lactamases) or quinolones (47.7% and 20.8%, respectively) (European Centre for Disease Prevention and Control 2015). It has also been frequently reported that *qnr* genes (encoding plasmid mediated quinolone resistance) are identified among isolates producing extended-spectrum beta-lactamases (ESBLs) (Nordmann and Poirel, 2005; Strahilevitz *et al.,* 2009).

Key words:
Wastewater, Enterobacteria, Broad-spectrum beta-lactamases, Genetic analysis.

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Another significant fact is the spread of antibiotic-resistant bacteria (ARB) or antibiotic-resistant genes (ARGs) in the environment and the release of large amounts of antibiotics into wastewater; both in metabolized or unmetabolized forms (Rizzo *et al.,* 2013; Bouki *et al.,* 2013). There is evidence that genes encoding resistance to all antibiotic classes were observed in wastewater treatment plant (WWTP). Therefore, WWTPs seem to be the main anthropogenic sources for antibiotics, ARB and ARGs into the environment (Rizzo *et al.,* 2013).
Wastewater from health care facilities may contain potentially dangerous components like microbiological pathogens, hazardous chemicals, pharmaceuticals or radioactive isotopes (World Health Organisation, 2000). Many hospitals have their own WWTPs in order to eliminate the physical, chemical or biological pollutants and reduce the impact of the effluent on the environment and human health.
In recent years, an increasing number of studies have demonstrated the presence of ARB, ARGs and unmetabolized antimicrobial residues in the effluent from hospital or urban WWTPs releasing these contaminants directly into the natural environment. Methicillin-resistant *Staphylococcus aureus* (MRSA) (Börjesson *et al.,* 2010; Rosenberg Goldstein *et al.,* 2012), ESBL-positive enterobacteria (Dolejská *et al.,* 2011; Diwan *et al.,* 2012; Ojer-Usoz *et al.,* 2014), quinolone-resistant strains (Akter *et al.,* 2012), as well as *vanA* (Kotzammanidis *et al.,* 2009; Araújo *et al.,*

2010), *ampC* (Szczepanowski *et al.,* 2009, Yim *et al.,* 2013) and *tet* genes (Szczepanowski *et al.,* 2009, Okoh and Igbinosa, 2010; LaPara *et al.,* 2011) have been detected in wastewater samples. Therefore, WWTPs could represent a significant natural reservoir in the development and dissemination of ARB (Martinez, 2009) and potential hotspots for antibiotic resistance gene transfer (Rizzo, 2013).

The aims of the study were to:

1) investigate the level of ARB in university hospital wastewater, focusing on broad-spectrum beta-lactamase-producing (ESBL and AmpC) and fluoroquinolone-resistant enterobacteria;
2) identify the genetic basis of resistance to beta-lactams (detection of *bla* genes) and plasmid-mediated fluoroquinolone resistance in selected bacteria;
3) determine the similarity/identity of isolates obtained from wastewater and from hospitalized patients;
4) monitor the level of ARB in urban wastewater.

MATERIALS AND METHODS

Sample collection - part 1

The first part of the study was conducted in the WWTP of Olomouc University hospital (H-WWTP) providing a full range of patient care services in all medical fields for the Olomouc Region. The facility comprising 52 departments and 1184 beds cares more than 50,000 inpatients each year. The H-WWTP uses a combination of mechanical and biological treatment processes with subsequent water disinfection using chlorine dioxide. Treated hospital wastewater effluent goes directly into the urban WWTP (U-WWTP) system.

Wastewater samples were collected in September 2013 and 2014. Cellulose swabs (length 11 cm, diameter 5 cm) were immersed into wastewater at the WWTP inflow and outflow as described by Moore *et al.* (Moore *et al.,* 1952). After 48-hour immersion, the swabs were inserted into a nutrient broth for 24 h and subsequently inoculated onto blood agar, MacConkey agar and desoxycholate citrate agar (Trios, Prague, Czech Republic).

Moreover, in September 2014, wastewater samples (a volume of 50 mL) were obtained directly from the final treated effluent using sterile plastic tubes. The samples were stored at 4°C during transportation to the laboratory and inoculated onto the above culture media.

Identification and characterization of bacterial strains

The obtained bacterial isolates were identified by the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) system (Biotyper Microflex, Bruker, Bremen, Germany). In all isolates, susceptibility to antibiotics was assessed by a standard microdilution method according to the European Committee on Antimicrobial Susceptibility Testing criteria (European Committee on Antimicrobial Susceptibility Testing, 2013). All bacteria with the minimum inhibitory concentration (MIC) of the tested 3rd and 4th generation cephalosporins of 1 mg/L or more were screened for ESBL and AmpC production. Determination of ESBL was performed by Jarlier's double-disc synergy test (DDST) which was modified by including a disc with cefepime and another disc with ceftazidime and ceftazidime/clavulanic acid (Jarlier *et al.,* 1988, Htotutou Sedláková *et al.,* 2011). AmpC-positive strains were identified by a modified AmpC disc test using 3-aminophenylboronic acid (Yagi *et al.,* 2005, Htoutou Sedláková *et al.,* 2011).

Sample collection - part 2

At the same times (in addition one week before and one week after wastewater sample collection), enterobacteria producing broad-spectrum beta-lactamases (ESBL or AmpC) were obtained from clinical samples of patients staying in all departments of the University hospital. From each patient, only one isolate of the particular species was included. Identification and determination of the production of broad-spectrum beta-lactamases was carried out as described previously.

Sample collection - part 3

The last part of the study was conducted in U-WWTP of the town of Olomouc. In September 2014, wastewater samples were collected at the inflow and outflow using the above procedure, with subsequent characterization of the obtained isolates. The U-WWTP uses a combination of mechanical and biological processes and treats domestic, industrial and health care wastewater of the town and the surrounding areas. The biological stage is designed as the so-called R-D-N system (regeneration-denitrification-nitrification) with post-denitrification and post-aeration systems; the effluent goes directly into the river. The U-WWTP capacity (population equivalent) is 295500.

Genetic detection of beta-lactamase genes and Qnr proteins

Polymerase chain reaction (PCR) was performed to characterize enzymes related to the three most widespread beta-lactamase families, TEM, SHV and CTX-M (Arlet *et al.,* 1995; Chanawong *et al.,* 2000; Pagani *et al.,* 2003). Specific sets of primers were used in order to amplify selected types of Qnr proteins in potential ESBL or AmpC producers (Cattoir *et al.,* 2001). Multiplex PCR was carried out to confirm the production of AmpC beta-lactamases (Pérez-Pérez and Hanson, 2003).

Pulsed-field gel electrophoresis (PFGE)

The epidemiological relationship of the isolates from wastewater and hospitalized patients was determined by PFGE. Genomic DNA was isolated from a bacterial culture incubated at 37°C for 18 h in Mueller-Hinton broth according to procedure a described by Husičková *et al.* (Husickova *et al.,* 2012). Total bacterial DNA was cleaved using specific enzymes, *Xba*I (Takara Bio, Otsu, Shiga, Japan) or *Spe*I (Takara Bio, Otsu, Shiga, Japan). Macrorestriction fragments were subsequently separated in 1.2% (w/v) Agarose gel (Pulsed Field Certified agarose, Bio-Rad, California, USA) after overflowing with 0.5 × TBE (Bio-Rad, California, USA). PFGE was carried out using the following parameters: run time 24 h at 14°C, pulse time 2 to 35 s and voltage 6 V/cm. After staining of the gel with ethidium bromide (1 µg/mL) (Sigma-Aldrich, Munich, Germany), the resulting restriction profiles were documented using an imaging device and compared by visual comparison and by the GelCompar II software (Applied Maths, Kortrijk, Belgium). The results were interpreted according to criteria described by Tenover *et al.* (Tenover *et al.,* 1995).

RESULTS

ESBL- and AmpC-producing enterobacteria in hospital wastewater samples

A total of 21 and 30 isolates of enterobacteria were obtained from wastewater samples in 2013 and 2014, respectively. The most frequently identified species were *Citrobacter freundii*, *K. pneumoniae* and *E. coli* (*Table 1*). Duplicates were removed on the basis of antibiograms and PFGE. The obtained data showed relatively high levels of bacterial resistance to 3rd generation cephalosporins (57.1% to cefotaxime and 61.9% to ceftazidime in 2013,

Table 1 - *Distribution of bacterial isolates from hospital, urban wastewater and clinical samples.*

		ORIGIN							
		Total no.	*H-WWTP*	*H-WWTP*	*H-WWTP*	*Clinical*	*U-WWTP*	*U-WWTP*	*U-WWTP*
Year	*Species*	*of isolates*	*Inflow*	*Outflow*	*Outflow-Water*	*samples*	*Inflow*	*Outflow*	*Outflow-Water*
2013	*Klebsiella pneumoniae*	7	4	3	-	-	-	-	-
	Escherichia coli	5	5	0	-	-	-	-	-
	Citrobacter freundii	4	2	2	-	-	-	-	-
	Morganella morganii	2	2	0	-	-	-	-	-
	Proteus mirabilis	2	1	1	-	-	-	-	-
	Enterobacter asburiae	1	0	1	-	-	-	-	-
	Klebsiella pneumoniae	33	-	-	-	33	-	-	-
	Escherichia coli	16	-	-	-	16	-	-	-
	Citrobacter freundii	3	-	-	-	3	-	-	-
	Morganella morganii	2	-	-	-	2	-	-	-
2014	*Citrobacter freundii*	10	3	3	4	-	-	-	-
	Klebsiella pneumoniae	5	3	1	1	-	-	-	-
	Serratia marcescens	3	1	2	0	-	-	-	-
	Enterobacter asburiae	3	1	1	1	-	-	-	-
	Citrobacter braakii	2	1	1	0	-	-	-	-
	Klebsiella oxytoca	2	2	0	0	-	-	-	-
	Enterobacter radicincitans	1	0	0	1	-	-	-	-
	Enterobacter kobeii	1	1	0	0	-	-	-	-
	Morganella morganii	1	1	0	0	-	-	-	-
	Proteus mirabilis	1	1	0	0	-	-	-	-
	Escherichia coli	1	1	0	0	-	-	-	-
	Klebsiella pneumoniae	37	-	-	-	37	-	-	-
	Escherichia coli	15	-	-	-	15	-	-	-
	Enterobacter cloacae	7	-	-	-	7	-	-	-
	Enterobacter koserii	2	-	-	-	2	-	-	-
	Citrobacter freundii	2	-	-	-	2	-	-	-
	Citrobacter koserii	1	-	-	-	1	-	-	-
	Citrobacter freundii	19	-	-	-	-	6	10	3
	Klebsiella pneumoniae	4	-	-	-	-	4	0	0
	Klebsiella oxytoca	4	-	-	-	-	2	2	0
	Raoultella ornithinolytica	3	-	-	-	-	2	0	1
	Enterobacter cloacae	2	-	-	-	-	0	2	0
	Enterobacter kobei	1	-	-	-	-	0	0	1
	Escherichia coli	1	-	-	-	-	0	1	0
	Klyuvera cryocrescens	1	-	-	-	-	0	0	1
	Providencia rettgeri	1	-	-	-	-	1	0	0
	Escherichia hermannii	1	-	-	-	-	0	1	0
	Proteus vulgaris	1	-	-	-	-	0	1	0

Table 2 - *Percentage of resistant isolates of single bacterial species isolated from wastewater samples.*

Species	*Origin*	*AMP*	*AMS*	*CZL*	*CRX*	*GEN*	*COT*	*COL*	*OXO*	*OFL*	*TET*	*AZT*	*PIP*	*PPT*	*CPR*	*CTX*	*CTZ*	*CPM*	*CPS*	*MER*	*CIP*	*TIG*	*TOB*	*AMI*
2013 - HWWTP																								
Citrobacter freundii	Inflow+Outflow	100	100	100	100	75	50	0	75	75	75	75	100	100	25	75	100	50	25	0	75	0	100	0
Enterobacter asburiae	Outflow	100	100	100	100	100	100	0	100	100	100	0	100	100	0	100	100	0	0	0	100	0	0	0
Escherichia coli	Inflow	80	0	40	20	20	60	0	40	20	60	20	40	0	20	20	20	20	0	0	20	0	20	0
Klebsiella pneumoniae	Inflow+Outflow	100	100	100	100	100	100	14	86	71	100	100	100	57	100	100	100	100	57	0	86	57	100	14
Morganella morganii	Inflow	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	100	100	100	0
Proteus mirabilis	Inflow+Outflow	100	0	100	0	0	0	100	0	0	100	0	0	0	0	0	0	0	0	0	0	100	0	0
2014 - HWWTP																								
Citrobacter braakii	Inflow+Outflow	100	50	100	100	50	50	0	50	50	100	0	100	50	100	100	100	0	0	0	50	0	100	0
Citrobacter freundii	Inflow+Outflow	100	100	100	80	90	70	10	100	100	90	20	100	70	90	90	90	10	20	0	100	50	100	10
Enterobacter asburiae	Inflow+Outflow	100	100	100	100	100	0	100	100	100	100	0	100	100	100	100	100	0	0	0	100	100	100	0
Enterobacter kobei	Inflow	100	0	100	100	0	0	100	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter radicincitans	Outflow	100	0	100	100	0	100	0	0	0	100	100	100	0	100	100	100	100	0	0	0	100	100	0
Escherichia coli	Inflow	100	100	100	100	100	100	0	100	100	100	100	100	0	100	100	100	100	0	0	100	0	100	0
Klebsiella oxytoca	Inflow	100	100	100	100	100	50	50	100	100	100	100	100	100	100	100	100	100	100	50	100	50	100	50
Klebsiella pneumoniae	Inflow+Outflow	100	100	100	100	80	100	0	80	60	100	100	100	33	100	100	100	80	20	0	80	80	100	0
Morganella morganii	Inflow	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	100	100	100	0
Proteus mirabilis	Inflow	100	0	100	0	0	100	100	100	100	100	0	0	0	0	0	0	0	0	0	100	100	0	0
Serratia marcescens	Inflow+Outflow	100	100	100	100	100	100	100	100	100	100	67	67	33	100	100	100	66.7	0	0	100	67	100	67
2014 - UWWTP																								
Citrobacter freundii	Inflow+Outflow	100	100	100	58	0	0	0	0	0	0	5	16	0	0	0	0	0	0	0	0	11	5.3	0
Enterobacter cloacae	Outflow	100	100	100	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter kobei	Outflow	100	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli	Outflow	100	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0
Escherichia hermanii	Outflow	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Klebsiella oxytoca	Inflow+Outflow	75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	Inflow	100	25	25	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0	0	0	25	0	0
Klyuvera cryocrescens	Outflow	100	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteus vulgaris	Outflow	0	0	0	0	0	0	100	0	0	100	0	100	0	0	0	0	0	0	0	0	100	100	0
Providencia retgerii	Inflow	100	100	100	0	0	0	100	0	0	100	0	0	0	0	0	0	0	0	0	0	100	100	0
Raoultella ornithinolytica	Inflow+Outflow	100	0	0	0	0	0	0	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: AMP - ampicillin; AMS - ampicillin+sulbactam; CZL - cefazoline; CRX - cefuroxime; GEN - gentamicin; COT - co-trimoxazole; COL - colistin; OXO - oxolinic acid; OFL - ofloxacin; TET - tetracycline; AZT - aztreonam; PIP - piperacillin; PPT - piperacillin+tazobactam; CPR - cefoperazone; CTX - cefotaxime; CTZ - ceftazidime; CPM - cefepime; CPS - cefoperazone +sulbactam; MER - meropenem; CIP - ciprofloxacin; TIG - tigecycline; TOB - tobramycin; AMI - amikacin.

and 46.7% to cefotaxime and 73.3% to ceftazidime in 2014). An increase in fluoroquinolone resistance was also reported. While in 2013, the resistance to fluoroquinolone antibiotics did not exceed 62.0% (57.1% for ofloxacin and 61.9% for ciprofloxacin), a year later, the rates reached 83.3% in case of ofloxacin and 86.7% in case of ciprofloxacin. Relatively high level of resistance was also observed for gentamicin (66.7% in 2013 and 80.0% in 2014) and tetracycline (85.7% in 2013 and 96.7% in 2014) (data not shown). The percentage of resistant isolates of single bacterial species is shown in *table 2*.

Production of ESBL and AmpC was determined in 23 isolates from hospital wastewater using the specific mDDST method (12 from outflow samples) and in 12 by the modified AmpC disc test (8 from outflow sample). Subsequently, PCR confirmed the presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and genes encoding plasmid-mediated AmpC types of enzymes (*Table 3*). However, some discrepancies occurred

Table 3 - *Numbers of bacterial isolates from hospital wastewater and their genetic characteristics.*

Year	*Species*	*No. of isolates*	*ESBL*	*AmpC*	*Beta-lactamases*	*qnr*
2013	*Klebsiella pneumoniae*	4	+	-	TEM, SHV, CTX-M	*qnrB*
		1	+	-	TEM, CTX-M	*qnrB*
		1	+	-	SHV, CTX-M	*qnrB*
		1	NT	NT	NT	NT
	Escherichia coli	1	+	-	TEM, CTX-M	*qnrB*
		4	NT	NT	NT	NT
	Citrobacter freundii	2	-	+	CIT, DHA	*qnrB*
		1	-	+	TEM, CIT	-
		1	NT	NT	NT	NT
	Morganella morganii	2	NT	NT	NT	NT
	Proteus mirabilis	2	NT	NT	NT	NT
	Enterobacter asburiae	1	-	+	DHA	*qnrB*
2014	*Citrobacter freundii*	1	+	+	TEM, CIT	-
		1	+	-	TEM, CIT	-
		2	-	+	TEM, CIT, DHA	-
		1	-	+	TEM, CIT	-
		1	-	+	CIT, DHA	*qnrB*
		1	-	-	TEM, CIT	-
		2	-	-	-	-
		1	NT	NT	NT	NT
	Klebsiella pneumoniae	2	+	-	TEM, SHV, CTX-M	*qnrB*
		2	+	-	TEM, CTX-M	*qnrB*
		1	-	+	SHV, DHA	*qnrB*
	Serratia marcescens	1	+	-	CTX-M	-
		1	+	-	TEM	-
		1	+	-	-	-
	Enterobacter asburiae	1	+	+	TEM, DHA, EBC	*qnrB*
		2	-	-	TEM, DHA, EBC	*qnrB*
	Citrobacter braakii	1	+	-	-	-
		1	+	-	TEM	-
	Klebsiella oxytoca	1	+	-	TEM, CTX-M	*qnrB*
		1	+	-	-	
	Enterobacter radicincitans	1	+	+	TEM, CTX-M	*qnrB*
	Enterobacter kobei	1	NT	NT	NT	NT
	Morganella morganii	1	NT	NT	NT	NT
	Proteus mirabilis	1	NT	NT	NT	NT
	Escherichia coli	1	+	-	CTX-M	*qnrB*

Note: NT - not tested (enterobacteria with the minimum inhibitory concentration of the tested 3rd and 4th generation cephalosporins less than 1 mg/L were not screened for ESBL and AmpC production).

between the phenotypic and genetic tests. In some samples, the presence of AmpC genes was not expressed as an AmpC phenotype and could not be detected by specific tests. In another three samples, another type of beta-lactamase could be present. Moreover, genetic determinants (*qnrB*) responsible for production of QnrB proteins were determined in 24 isolates.

ESBL - and AmpC-producing enterobacteria in urban wastewater samples

Over the study period, a total of 38 enterobacteria were collected from the U-WWTP. The results obtained are summarized in *Table 1*. The level of bacterial resistance to 3rd generation cephalosporins did not exceed 11.0% (2.6% for cefotaxime and 10.5% for ceftazidime). All isolates showed susceptibility to ciprofloxacin. In relation to other classes of antimicrobial agents, a reduced number of resistant isolates was observed as compared to hospital wastewater samples (0% for gentamicin, 0% both for ofloxacin and ciprofloxacin and 8.3% for tetracycline). The percentage of resistant isolates of single bacterial species is shown in *table 2*. In contrast to hospital wastewater samples, no ESBL or even AmpC phenotypes were confirmed by the mDDST and modified AmpC test. However, five isolates were screened as potential producers of broad-spectrum beta-lactamases. No *qnr* genes were detected in the analyzed isolates.

ESBL - and AmpC-producing enterobacteria in hospitalized patients

A total 54 and 64 enterobacteria with production of broad-spectrum beta-lactamases were obtained from patients staying in Olomouc University Hospital in 2013 and 2014, respectively (*Table 1*). The most frequent clinical specimens were urine, stool and lower respiratory tract samples. The patients' ages spanned 0-91 years and they were admitted to different hospital departments (hemato-oncology, respiratory-medicine, surgery, urology, neurology, internal medicine, pediatrics, neonatology, dermatology and venerology, anesthesiology and intensive care medicine, neurosurgery, exercise medicine and cardiovascular rehabilitation, orthopedics and geriatrics).

Genetic relationship of collected isolates

Isolates of *E. coli*, *C. freundii*, *K. pneumoniae* and *M. morganii* from wastewater and patients were compared by PFGE (*Figures 1-3*; data for *M. morganii* are not shown). This method revealed several groups of identical isolates in hospitalized patients, especially in the species of *K. pneumoniae*. Some samples (two *C. freundii* and two *E. coli*) had to be removed from the final analysis because of problematic DNA isolation.

The most important finding was the detection of two identical isolates (OPV1-71 and C9) of *K. pneumoniae* in 2013 (*Figure 3*), one from a patient's urinary catheter and the other from wastewater at the H-WWTP inflow. On the other hand, no genetic relationship between the 2014 hospital and wastewater isolates was determined. Bacterial strains of *K. pneumoniae*, *E. coli* and *C. freundii* from the U-WWTP showed no genetic similarity with either patient or with hospital wastewater samples.

DISCUSSION

Antibiotic resistance has probably developed as a mechanism of self-protection of antibiotic-producing microbes to avoid self-destruction during the production of agents with antimicrobial activity (Walsh, 2003). However, there is evidence that exposure to non-antibiotic agents like biocides and heavy metals may induce or select for decreased susceptibility to antibiotics (Martínez, 2008; Walsh and Davies, 2015).

Antibiotic resistance is a natural phenomenon but currently represents a very serious problem and a significant threat because of the widely spread resistant bacteria in healthcare settings and infections caused by these microbes.

WWTPs are systems for improving the quality of wastewater before it is discharged to the aquatic environment, mainly to the rivers. The treatment process usually consists of primary (mechanical), secondary (biological) and tertiary treatment. The aim of these processes is to reduce suspended solids, biodegradable organics, pathogenic bacteria and nutrients (including nitrates and phosphates) (World Bank Group, 2014).

Recent studies from various countries have pointed to the presence of ARB, ARGs as well as antibiotics in sewage, activated sludge, sediments, and hospital and urban wastewater effluent (Martinez, 2009; Akiyama and Savin, 2010; Munir *et al.,* 2011, Huang *et al.,* 2012; Ottosson *et al.,* 2012; Kotzammanidis *et al.,* 2009; Börjesson *et al.,*

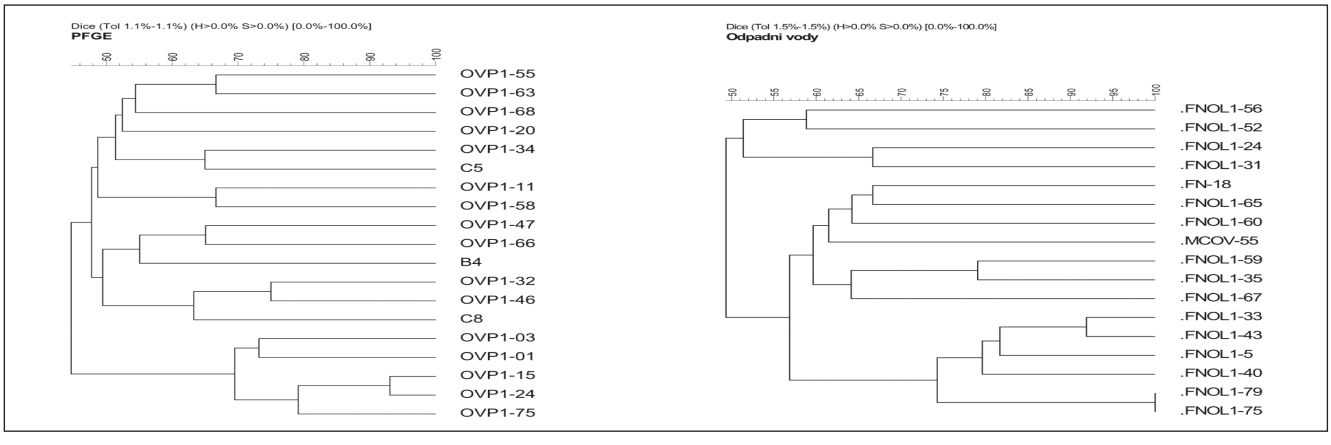

Figure 1 - *Dendrogram of E. coli isolates collected in 2013 and 2014. Horizontal axis - similarity of isolates (%). Vertical axis - isolate names. (OVP1- isolates from inpatients in 2013; C5, B4, C8 - isolates from H-WWTP in 2013). (FNOL1- isolates from inpatients in 2014; FN - isolates from H-WWTP in 2014, MCOV - isolates from U-WWTP in 2014).*

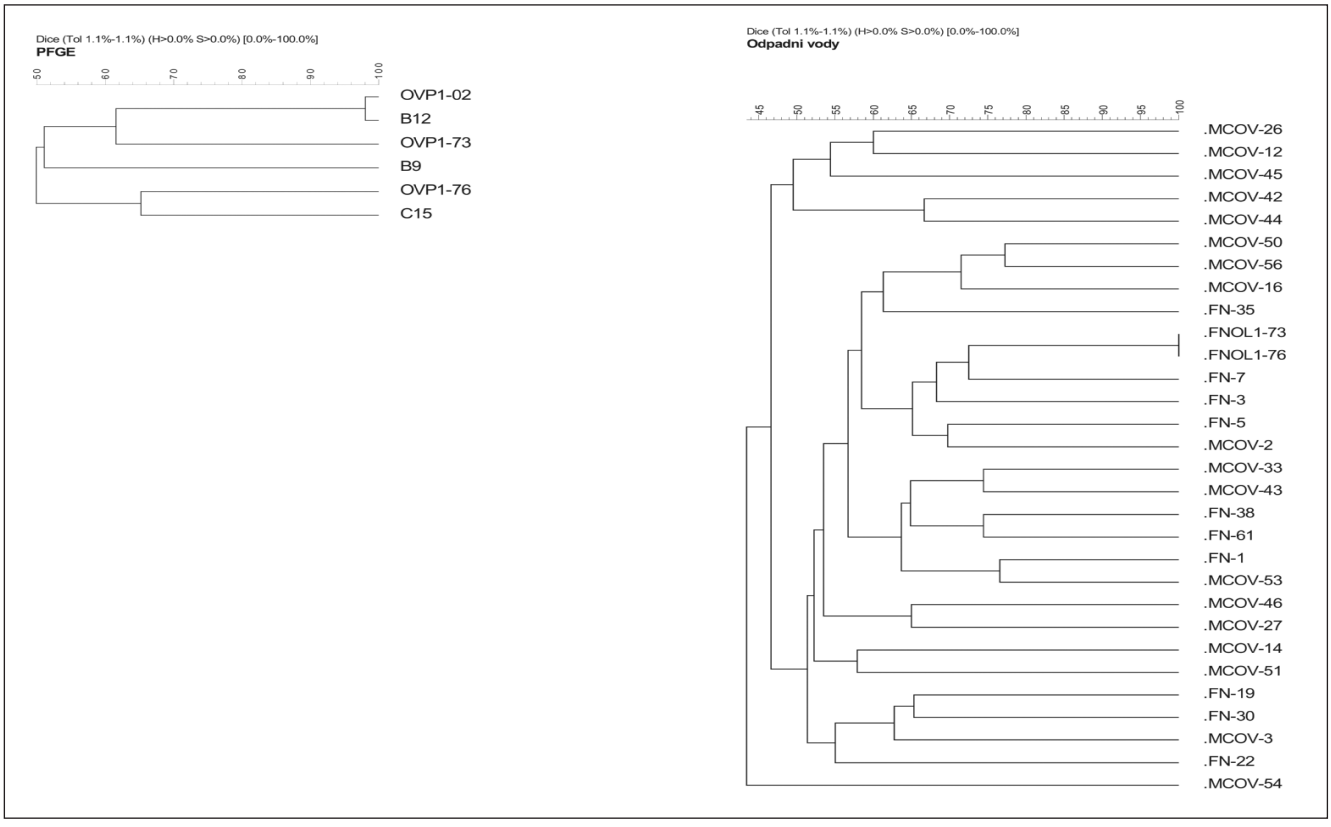

Figure 2 - *Dendrogram of C. freundii isolates collected in 2013 and 2014. Horizontal axis - similarity of isolates (%). Vertical axis - isolate names. (OVP1 - isolates from inpatients in 2013; B12, B9, C15 - isolates from H-WWTP in 2013).*
(FNOL1 - isolates from inpatients in 2014; FN - isolates from H-WWTP in 2014, MCOV - isolates from U-WWTP in 2014).

2010). There is evidence that genes encoding resistance to all antibiotic classes were observed in WWTPs (Rizzo *et al.,* 2013). The authors warn that WWTP systems may act as a source of resistance genes with potential spread to the environment. However, the presence of antibiotics as a driving force in wastewater remains a matter of debate (Bouki *et al.,* 2013).

Based on these findings we decided to investigate the levels of antimicrobial-resistant enterobacteria in university hospital wastewater focusing on broad-spectrum beta-lactamase (ESBL and AmpC) producers. In addition, comparing to above mentioned studies, we analyzed the similarity/identity of isolates obtained from wastewater and from hospitalized patients. The pilot study started in September 2013 with the first part of the experiments. In the next year, an extended study was performed including a greater spectrum of analyzed samples. Moreover, wastewater samples were also collected from an U-WWTP due to the fact that treated hospital effluent goes directly into this system. In the present study, a total of 51 isolates of enterobacteria were obtained from hospital wastewater samples. Overall, 45.1% of them produced broad-spectrum beta-lactamases (ESBL or AmpC enzymes according to the phenotypic tests). Enterobacteria from the U-WWTP displayed lower levels of antibiotic resistance. Only 5 isolates were indicated as potential ESBL or AmpC producers according to the MIC values. Nevertheless, the specific tests did not confirm the above phenotypes.

A study by Ojer-Usoz *et al.* (Ojer-Usoz *et al.,* 2014) analyzed 279 effluent samples from 21 WWTPs in northern Spain. Among 185 isolates of enterobacteria obtained from effluent water and cultured on the chromID ESBL medium, 163 (88.1%) were classified as ESBL-positive. In another study, 68 isolates (60.0%) of enterobacteria collected from treated water U-WWTP were indicated as ESBL producers (Dolejská *et al.,* 2011). In a group of 310 isolates collected from three hospitals in Olsztyn, Poland, 150 (48.4%) were confirmed as ESBL producers according to phenotypic detection tests (Korzeniewska and Harnisz, 2013a). In the same year, the authors published another study focused on the detection of ESBL-positive enterobacteria in municipal sewage. A total number of 455 collected strains (from sewage samples, river water and air samples collected at the WWTP area) were analyzed. The results demonstrated 100% prevalence of ESBL-producing enterobacteria in sewage samples, 33.3% in river water and 23.8% in air samples (Korzeniewska and Harnisz, 2013b).

The second part of the present study focused on the detection of a genetic basis of resistance to beta-lactams (detection of *bla* genes) and plasmid-mediated fluoroquinolone resistance in selected bacteria. TEM, SHV, CTX-M as well as CIT and DHA types of beta-lactamases were detected in the 2013 pilot study. In some samples, combinations of three types of enzymes were assessed (TEM, SHV and CTX-M in four *K. pneumoniae* isolates and TEM, CIT and DHA in two *C. freundii* samples). In the next year, apart from the above beta-lactamases, EBC enzymes were also detected. In 2014, six isolates with combinations of three types of beta-lactamases were identified (two isolates of *C. freundii* with TEM, CIT, DHA; two isolates of *K. pneumoniae* with TEM, SHV, CTX-M and three samples of *E. asburiae* with a combination of TEM, DHA and EBC enzymes).

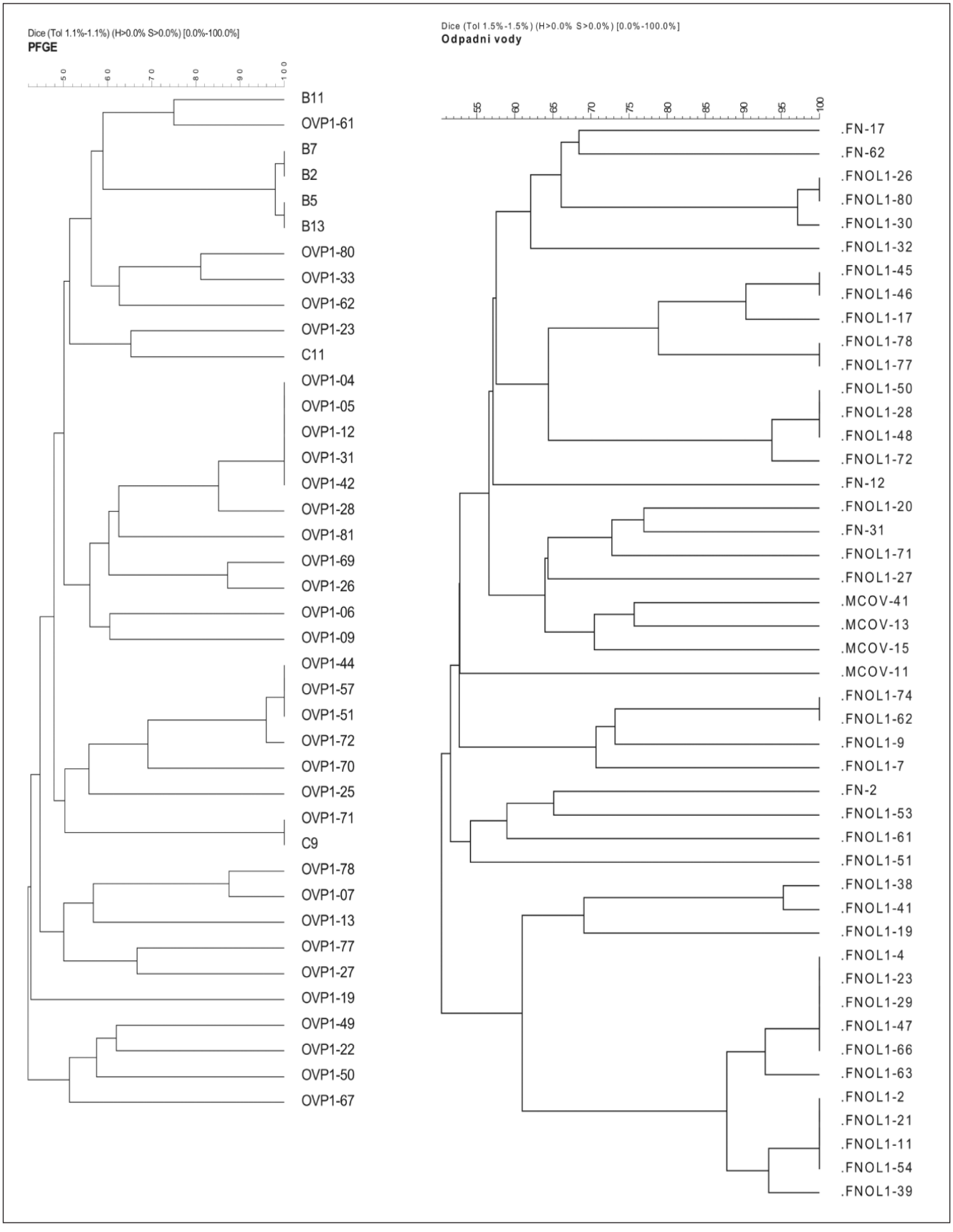

Figure 3 - *Dendrogram of K. pneumoniae isolates collected in 2013 and 2014.*
Horizontal axis - similarity of isolates (%). Vertical axis - isolate names.
(OVP1 - isolates from inpatients in 2013; B11, B7, B2, B5, C11, C9 - isolates from H- WWTP in 2013).
(FNOL1 - isolates from inpatients in 2014; FN - isolates from H-WWTP in 2014, MCOV - isolates from U-WWTP in 2014).

In the present study, bacterial isolates containing bla_{TEM}, bla_{SHV}, bla_{CTX-M} or genes encoding CIT, DHA, EBC enzymes and Qnr proteins were collected from the hospital WWTP effluent, representing a potential environmental threat. However, the presence of these genetic determinants did not always correspond to an ESBL or ampC phenotype. Sometimes, enzymes with narrow-spectrum activity could be assessed (SHV-1, TEM-1 or TEM-2) (Bradford, 2001). On the other hand, the expression of the chromosomal *ampC* gene is known to be low but inducible in many Gram-negative bacteria including *C. freundii* (Hansen and Sanders, 1999).

Therefore, some discrepancies between the phenotypic and genetic methods may occur. Moreover, some samples (one *S. marcescens*, one *C. braakii* and one *K. oxytoca*) probably contained genes encoding other than tested types of beta-lactamases that were not detected.

High prevalence rates of *bla* genes were determined in two studies from Poland (Korzeniewska and Harnisz, 2013a, Korzeniewska and Harnisz, 2013b). From all ESBL producers collected from municipal sewage samples, river water and air samples, 61.0% possessed $bla_{CTX-M-1-like}$, 26.8% $bla_{CTX-M-9-like}$, 29.3% bla_{SHV} and 26.8% bla_{TEM} genes. Almost 44% carried several genes. By contrast, the majority of 150 ESBL-positive enterobacteria from hospital effluents harbored $bla_{CTX-M-3}$, followed by different types of CTX-M, SHV and TEM beta-lactamases. Twenty-two isolates were found to contain two or three *bla* genes. In a study from Brazil, Chagas *et al.* (Chagas *et al.,* 2011) examined wastewater samples from hospital WWTP in Rio de Janeiro and detected $bla_{TEM,}$ bla_{SHV} and bla_{CTX-M} genes in 82.0%, 48.0% and 67.0% of bacterial isolates, respectively. Our study demonstrated the presence of genes encoding TEM, SHV and CTX-M beta-lactamases in 57.1%, 19.0% and 33.3% isolates, respectively, collected from the hospital WWTP outflow in both years.

Plasmid-mediated quinolone resistance determinants (*qnr A*, *B*, and *S*) were detected in ciprofloxacin-resistant enterobacteria from sludge and from raw sewage in Israel (Kaplan *et al.,* 2013). In the study of Yim G (Yim *et al.,* 2012), complex integrons containing *qnr*B4-*ampC* -(bla_{DHA-1}) were determined in multidrug resistant *C. freundii* form wastewater. Dolejská et al. (Dolejská *et al.,* 2011) identified *qnrB1* in CTX-M-15 producing *K. pneumoniae* isolates from municipal WWTP effluent in Brno, in the Czech Republic. In our study, 68.6% of broad-spectrum producing enterobacteria from hospital wastewater samples harboured *qnrB* type of plasmid-mediated quinolone resistance determinant.

In conclusion, multiresistant enterobacteria producing ESBL or AmpC type of broad-spectrum beta-lactamases were identified both at the inflow as well as outflow of H-WWTP. The most important finding was the detection of two identical isolates of *K. pneumoniae* in 2013, one from a patient's urinary catheter and the other from a wastewater sample.

Given the fact that healthcare facility wastewater may act as a source of resistance genes with potential spread to environment, treatment as well as disinfection procedures must be improved to reduce the potential environmental spread of resistant bacteria.

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Conflict of interest

The authors declare no conflict of interest.

References

Akiyama T., Savin M.C. (2010). Populations of antibiotic-resistant coliform bacteria change rapidly in a wastewater effluent dominated stream. *Sci. Total Environ.* **408**, 6192-6201.

Akter F., Amin M.R., Osman K.T., Anwar M.N., Karim M.M., Hossain M.A. (2012). Ciprofloxacin-resistant *Escherichia coli* in hospital wastewater of Bangladesh and prediction of its mechanism of resistance. *World J Microbiol Biotechnol.* **28**, 827-834.

Araújo C., Torres C., Silva N., Carneiro C., Gonçalves A., Radhouani H., et al. (2010). Vancomycin-resistant enterococci from Portuguese waste water treatment plants. *J Basic Microbiol.* **50**, 605-609.

Arlet G., Brami G., Décrè D., Flippo A, Gaillot O., Lagrange P.H., Philippon A. (1995). Molecular characterisation by PCR-restriction fragment length polymorphism of TEM beta-lactamases. *FEMS Microbiol. Lett.* **134**, 203-208.

Bouki C., Venieri D., Diamadopoulos E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatmen
t plants: A review. *Ecotox Environ Safe.* **91**, 1-9.

Börjesson S., Matussek A., Melin S., Löfgren S., Lindgren P.E. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: an uncharted threat? *J Appl Microbiol.* **108**, 1244-1251.

Bradford P.A. (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev.* **14**, 933-951.

Cattoir V., Poirel L., Nordmann P. (2001). Multiplex PCR for detectíon of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. *J Antimicrob Chemother.* **60**, 394-391.

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

Chanawong A., M'Zali F.H., Heritage J., Lulitanond A., Hawkey P.M. (2000). Characterisation of extended-spectrum beta-lactamases of the zpolymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP). *FEMS Microbiol Lett.* **184**, 85-89.

Diwan V., Chandran S.P., Tamhankar A.J., Stålsby Lundborg C., Macaden R. (2012). Identification of extended-spectrum beta-lactamase and quinolone resistance genes in *Escherichia coli* isolated from hospital wastewater from central India. *J Antimicrob Chemother.* **67**, 857-859.

Dolejska M., Frolkova P., Florek M., Jamborova I., Purgertova M., Kutil I., et al. (2011). CTX-M-15 producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella* spp. isolates in municipal wastewater treatment plant effluents. *J Antimicrob Chemother.* **66**, 2784-2790.

EUCAST. (2013). European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zonediameters [online]. Available from <http://www.eucast.org/clinical_breakpoints>[accessed on 02 September 2012].

European Centre for Disease Prevention and Control. (2015). Antimicrobial resistance interactive database (EARS-Net) [online]. Available from <http://www.ecdc.europa.eu/en/Pages/home.aspx >. [accessed on 10 May 2015].

Hanson N.D, Sanders C.C. (1999). Regulation of inducible AmpC beta-lactamase expression among *Enterobacteriaceae*. *Curr Pharm Des.* **5**, 881-894.

Htoutou Sedlakova M., Hanulik V., Chroma M., Hricova K, Kolar M., Latal T., et al. (2011) Phenotypic detection of broad-spectrum beta-lactamases in microbiological practice. *Med Sci Monitor.* **17**, BR 147-52.

Huang J.J., Hu H.Y., Lu, S.Q., Tang, F., Lu, Y., Wei B. (2012). Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. *Environ Int.* **42**, 31-36.

Husičková V., Čekanová L., Chromá M., Htoutou-Sedlakova M, Hricova K, Kolar M. (2012). Carriage of ESBL- and AmpC-positive *Enterobacteriaceae* in the gastrointestinal tract of community subjects and hospitalized patients in the Czech Republic. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. **156**, 348-353.

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

Kaplan E., Ofek M., Jurkevitch E., Cytryn E. (2013). Characterizaction of fluoroquinolone resistence and *qnr* diversity in *Enterobacteriaceae* from municipal biosolids. *Front Microbiol.* **4**, 144.

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

Korzeniewska E., Harnisz M. (2013b). Extended-spectrum beta-lactamase (ESBL)-positive *Enterobacteriaceae* in municipal sewage and their emission to the environment. *J Environ Manage.* **128**, 904-911.

Kotzammanidis C., Zdragas A., Kourelis A., Moraitou E., Papa A., Yiantzi V., et al. (2009). Characterization of vanA-type *Enterococcus faecium* isolates from urban and hospital wastewater and pigs. *J Appl Microbiol.* **107**, 997-1005.

LaPara T.M., Burch T.R., McNamara P.J., Tan D.T., Yan M., Eichmiller J.J. (2011). Tertiary treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environ Sci Technol. **45**, 9543-9549.

Martínez J.L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science.* **18**, 365-367.

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

Moore B., Perry E.L., Chard S.T. (1952). A survey by the sewage swab method of latent enteric infections in an urbanarea. *J Hyg.* **50**, 137-156.

Munir M., Wong K., Xagoraraki I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* **45**, 681-693.

Nordman P., Poirel L. (2005). Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. *J Antimicrob Chemother.* **56**, 463-469.

Ojer-Usoz E., Gonzáles D., García-Jalón I., Vitas A.I. (2014). High dissemination of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in effluents from wastewater treatment plants. *Water Res.* **56**, 37-47.

Okoh A.I., Igbinosa E.O. (2010). Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rular community of the Eastern Cape Province of South Africa. *BMC Microbiol.* **10**, 143-148.

Ottosson J.R., Jarnheimer P.A., Stenström T.A. Olsen B. (2012). A longitudinal study of antimicrobial resistant faecal bacteria in sediments collected from a hospital wastewater system. *Infect Ecol and Epidemiol.* **2**, 7438-7444.

Pagani L., Dell'Amico E., Migliavacca R., D'Andrea M.M., Giacobone E., et al. (2003). Multiple CTX-M-type extended-spectrum beta-lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in northern Italy. *J Clin Microbiol.* **41**, 4264-4269.

Pérez-Pérez F.J., Hanson N.D. (2002). Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol.* **40**, 2153-2162.

Rizzo L., Manaia C., Merlin C., Schwartz T., Dagot C., Ploy M.C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci Total Environ.* **447**, 345-360.

Rosenberg Goldstein E.E., Micallef S.A., Gibbs S., Davis A., He X., George A., et al. (2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environ Health Pers.* **120**, 1551-1558.

Strahilevitz, J., Jacoby G.A., Hooper D.C., Robicsek A. (2009). Plasmid-mediated quinolone resistance: A multifaceted treat. *Clin Microbiol Rev.* **22**, 664-689.

Szczepanowski R., Linke B., Krahn I., Gartemann K.H., Gűtzkow T., Eichler W., et al. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of waste water treatment plant bacteria showing reduces susceptibility to selected antibiotics. *Microbiology.* **55**, 2306-2319.

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B., Persing D.H., Swaminathan B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis:criteria for bacterial strain typing. *J Clin Microbiol.* **33**, 2233-2239.

Wales D.A., Davies R.H. (2015). Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. **4**, 567-604.

Walsh C. (2003). Antibiotics: action, origins, resistance; ASM Press: Washington D.C.

World Bank Group. 2014. Introduction *to* water treatment processes [online]. Available from <http://water.worldbank.org/shw-resource-guide/infrastructure/menu-technical-options/wastewater-treatment > [accessed on 02 December 2014].

World Health Organisation. (2000). Collection and disposal of wastewater[online]. Available from <http://www.who.int/water_sanitation_health/medicalwaste/130to134.pdf> [accessed on 10 November 2013].

Yag T., Wachino, J., Kurokawa H., Suzuki S., Yamane K., Doi Y., et al. (2005). Practical methods using boronic acid compounds for identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *J Clin Microbiol.* **43**, 2551-2558.

Yim G., Kwong W. Davies J. Miao V. (2013). Complex integrons containing *qnrB4-ampC* (bla_{DHA-1}) in plasmids of multidrug-resistant *Citrobacter freundii* from wastewater. *Can J Microbiol.* **59**, 110-116.