Journal of Antimicrobial Chemotherapy Advance Access published September 27, 2011

J Antimicrob Chemother
doi:10.1093/jac/dkr363

Journal of Antimicrobial Chemotherapy

CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella* spp. isolates in municipal wastewater treatment plant effluents

Monika Dolejska[1,2]*, Petra Frolkova[1], Magdalena Florek[1,3], Ivana Jamborova[1], Michaela Purgertova[1], Iva Kutilova[1], Alois Cizek[2,4], Sebastian Guenther[5] and Ivan Literak[1,2]

[1]Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1-3, 612 42 Brno, Czech Republic; [2]CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1-3, 612 42 Brno, Czech Republic; [3]Department of Microbiology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, C.K. Norwida 30, 50-375 Wroclaw, Poland; [4]Institute of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1-3, 612 42 Brno, Czech Republic; [5]Institute of Microbiology and Epizootics, Veterinary Faculty, Free University Berlin, Philippstr. 13, 10115 Berlin, Germany

*Corresponding author. Tel: +420-541-562-643; Fax: +420-541-562-631; E-mail: m.dolejska@centrum.cz

Received 29 July 2011; returned 3 August 2011; revised 10 August 2011; accepted 11 August 2011

Objectives: The global occurrence of antibiotic resistance genes in bacteria in water environments is an increasing concern. Treated wastewater was sampled daily over a 45 day period from the outflow of a municipal wastewater treatment plant in Brno, Czech Republic, and examined for extended-spectrum β-lactamase (ESBL)-producing bacteria.

Methods: Water samples were cultivated on MacConkey agar with cefotaxime (2 mg/L) and individual colonies were examined for ESBL production. Phenotypic ESBL-positive bacteria identified as *Escherichia coli* or *Klebsiella* spp. were tested for the presence of antibiotic resistance genes, the virulence gene *afa/dra* and the bla_{CTX-M} upstream region. Genetic relatedness was analysed by PFGE, multilocus sequence typing and plasmid analysis.

Results: A total of 68 ESBL-producing Enterobacteriaceae isolates were detected in 34 out of 45 wastewater samples. ESBL-producing isolates included 26 *E. coli* isolates, 4 *Klebsiella pneumoniae* isolates and 1 *Klebsiella oxytoca* isolate. The pandemic and multiresistant B2-O25b-ST131 clone was predominant, being detected among 19 *E. coli* isolates, and 17 of the B2-O25b-ST131 isolates were positive for the FIA replicon and the *afa/dra* operon and had an IS*26* element flanking $bla_{CTX-M-15}$. Seventeen of the B2-O25b-ST131 isolates showed closely related PFGE profiles (defined by 84% band similarity) and belonged to identical clonal groups.

Conclusions: The results highlight the inadequacy of the treatment process in removing multiresistant bacteria from municipal wastewater and point to a risk of transmission of clinically important multiresistant strains, such as the pandemic ST131 clone, to the environment. This is the first study demonstrating the pandemic ST131 clone in wastewater.

Keywords: antibiotic resistance, MLST, plasmids

Introduction

The global occurrence of antibiotic resistance genes in bacteria in water environments is an increasing concern. Microorganisms that carry genes encoding resistance to a broad range of antibiotics have been found in hospital wastewater and animal production wastewater as well as in sewage, wastewater treatment plant (WWTP) effluents, surface water, river water, groundwater and drinking water.[1] Aquatic environments are described as natural reservoirs of antibiotic-resistant bacteria, and WWTPs are among the leading water reservoirs of these microorganisms.[2] Conditions such as pH, temperature, nutrient concentration and high bacterial biomass, favouring close contact of bacterial cells, make WWTPs ideal locations for gene transfer and the spread of resistance. In addition, the presence of antibiotics and their metabolites in sewage may promote both the selection of resistant strains and the horizontal transfer of antibiotic resistance genes.[3]

Wastewater treatment does not eliminate all pathogens and resistant bacteria.[2] Consequently, introducing treated wastewater effluents into natural water resources escalates the risk

of human exposure to resistant bacteria via drinking water systems[4] and the dissemination of these strains into wildlife.[4]

Emerging extended-spectrum β-lactamase (ESBL)-producing and fluoroquinolone-resistant bacteria have become a main issue in current human medicine. In 2008, worldwide pandemic spread of *Escherichia coli* sequence type (ST) 131, which produces CTX-M β-lactamase and is resistant to fluoroquinolones, was identified.[5] The clone belongs to pathogenic group B2 and mainly causes urinary tract infections in humans. Strains of this ST harbour a broad range of virulence and resistance genes on a transferable plasmid, most of which are of the IncF group.[6] While broad distribution has been demonstrated among antimicrobial-resistant *E. coli* from humans[7] our knowledge of the dissemination of ST131 remains limited.

The aim of this study was to isolate ESBL-producing and plasmid-mediated fluoroquinolone-resistant bacteria from treated water at a municipal WWTP with particular attention to *E. coli* and *Klebsiella pneumoniae*. To our knowledge, this was the first study investigating the occurrence of the multiresistant epidemiological uropathogenic clone *E. coli* B2-O25b-ST131 in municipal wastewater.

Materials and methods

Sampling

The municipal WWTP used for this study is situated in Brno, the second largest city in the Czech Republic, with a population of about 400000. The WWTP is a two-stage facility using a combined mechanical and biological treatment process with anaerobic sludge stabilization. It serves most districts of the city and its suburban areas, including several hospitals and long-term care facilities. Treated wastewater was sampled at the outflow of the WWTP, from which the effluents go directly into the Svratka River. The water samples were taken daily (except weekends) over a 45 day period between 26 November 2008 and 2 February 2009. The samples were taken using the Moore swab method.[8] The cotton swabs were exposed to the water effluents for 24 h, after which the swabs were collected, placed into sterile bags, then transported to the laboratory.

Isolation of ESBL-producing Enterobacteriaceae and antibiotic susceptibility testing

Wastewater samples diluted to 10^{-3} were cultured on MacConkey agar (MCA; Oxoid, UK) with cefotaxime (2 mg/L) to isolate ESBL-producing bacteria. All colonies showing differing morphology were selected from each plate, identified using the API 10S test kit (bioMerieux, France) and tested for ESBL production by the double-disc synergy test and for susceptibility to 12 antimicrobial agents according to the CLSI method,[9] as described previously.[10] Colonies identified as *E. coli* or *Klebsiella* spp. were further characterized.

Antibiotic resistance gene testing

PCR and sequencing were used to test for the presence of bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{OXA} genes in the ESBL-positive *E. coli* and *Klebsiella* spp. isolates.[11] Isolates resistant to additional antibiotics (aminoglycosides, chloramphenicol, sulphonamides and tetracycline) were tested for selected antibiotic resistance genes by PCR. For a list of primers and positive controls see Table S1 (available as Supplementary data at *JAC* Online). Isolates were screened for the presence of integrons; the genes *int1* and *int2*, the variable region of the class 1 integron, the variable region of the class 2 integron and the gene cassettes *dfr1*, *dfr12*, *dfr17*, *aadA1*, *aadA2*, *aadA5*, *estX* and *sat1/2* were tested for by PCR (Table S1). MICs of ciprofloxacin and nalidixic acid were determined for all strains using the CLSI agar dilution method[9] and strains were tested for the plasmid-mediated quinolone resistance genes *aac(6′)-Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* by PCR and sequencing.[10]

Upstream region of $bla_{CTX\text{-}M}$ genes

The genetic context of $bla_{CTX\text{-}M}$ genes was investigated by testing for the presence of and linkage with sequences previously reported to be associated with the CTX-M group genes, such as upstream regions IS*Ecp1* and IS*26*. PCR and sequencing using previously described primers were employed to investigate these surrounding regions. The IS*Ecp1* region was searched using IS*Ecp1* 5′ primer (5′-TTCAAAAAGCATAATCAAAG CC-3′)[12] binding in the 5′ region of IS*Ecp1* and using IS*Ecp1* UP primer (5′-AAAAATGATTGAAAGGTGGT3′)[13] binding to the transposase gene *tnpA* of IS*Ecp1*, located in the immediate $bla_{CTX\text{-}M}$ upstream region. The presence of IS*26* linked to $bla_{CTX\text{-}M}$ was tested using *tnpA* IS*26* primer (5′-AGCGGTAAATCGTGGAGTGA-3′),[12] recognizing the transposase gene of the IS*26* element. All primers were used in combination with reverse primer CTX-M-RCJ (5′-AGCGGCACACTTCCTAAC-3′), recognizing the 5′ region of $bla_{CTX\text{-}M}$ genes.[14]

Phenotypic and molecular typing methods

Identification of *E. coli* phylogenetic groups was performed using a multiplex PCR assay.[15] All isolates were subjected to serotyping.[16] Allele-specific PCR was performed to identify the O25-ST131 clone of *E. coli*.[17] The presence of replicons was tested by multiplex PCR.[18] *E. coli* and *Klebsiella* spp. isolates were typed by XbaI PFGE.[19] Profiles were analysed using BioNumerics fingerprinting software (Applied Maths, Belgium). Cluster analysis of the Dice similarity indices was done to generate a dendrogram describing the relationships among PFGE profiles. Isolates were considered to be related and to belong to the same PFGE cluster if their Dice similarity index was ≥80%. Multilocus sequence typing (MLST) was carried out as described previously.[20] Gene amplification and sequencing were performed using primers specified at the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). Sequences were analysed using the software package Ridom SeqSphere 0.9.19 (http://www3.ridom.de/seqsphere) and STs were computed automatically. MLST typing of *K. pneumoniae* strains was performed using the Institute Pasteur (Paris, France) scheme.[21]

Conjugation and transformation experiments

Conjugative transfer of ESBL genes was tested. Plate-mating experiments were performed using plasmid-free, rifampicin- and sodium azide-resistant *E. coli* and *Salmonella* recipients.[22] The strains were grown to the exponential phase and then mixed (1:1), and 500 μL of the donor and recipient mixture was incubated using a bacteriological filter on the surface of Luria–Bertani (LB) agar at 37°C overnight. Transconjugants were selected on LB agar supplemented with rifampicin, nalidixic acid and cefotaxime. Plasmid DNA from *E. coli* was isolated by the alkaline extraction procedure[23] and introduced to competent *E. coli* DH5α (Invitrogen, USA) by chemical transformation followed by selection of transformants on LB agar (Difco, USA) supplemented with cefotaxime (2 mg/L). The presence of the relevant *bla* gene in transformants and transconjugants was confirmed by PCR. Co-transfer of other antibiotic resistance genes during transformation and conjugation was tested by the disc diffusion test followed by PCR.

Plasmid characterization

The size of plasmids with ESBL genes from transformants or transconjugants was determined by S1-PFGE.[24] Plasmid DNA from transformants or transconjugants was digested with EcoRV, followed by gel electrophoresis in 1% agarose gel for 16 h at 67 V/cm. Plasmids were replicon typed.[18,25]

Plasmids belonging to incompatibility group FIIK were typed using a plasmid MLST scheme.[25]

Results

Isolation of cefotaxime-resistant and ESBL-producing Enterobacteriaceae

Forty-five water samples were taken between November 2008 and February 2009. A total of 114 cefotaxime-resistant Enterobacteriaceae isolates were obtained by incubation on MCA with cefotaxime (2 mg/L). Apart from resistance to β-lactam antibiotics, cefotaxime-resistant isolates showed resistance to nalidixic acid (90% of the isolates), followed by resistance to ampicillin/clavulanic acid and sulphonamides (46%), ciprofloxacin (45%), tetracycline (44%), sulfamethoxazole/trimethoprim (39%), streptomycin (27%), gentamicin (12%) and chloramphenicol (10%). ESBL production was detected in 68 (60%) Enterobacteriaceae isolates. At least one ESBL-producing isolate was detected in 34 (76%) out of 45 wastewater samples. ESBL producers included 26 isolates of *E. coli*, 4 isolates of *K. pneumoniae* and 1 isolate of *Klebsiella oxytoca*.

Determination of phenotypic and genotypic resistance in E. coli isolates

From the total of 26 ESBL-producing *E. coli* isolates, the gene $bla_{\text{CTX-M-15}}$ was detected in 23 (88%) isolates, while $bla_{\text{CTX-M-14b}}$ and $bla_{\text{CTX-M-1}}$ were detected in 1 and 2 isolates, respectively. All the isolates were multiresistant (resistant to two or more antibiotic groups); resistance to nalidixic acid and ciprofloxacin was the most prevalent (26 isolates), followed by sulphonamides (21), sulfamethoxazole/trimethoprim (19), tetracycline (19) and streptomycin (8) (Figure 1). In 21 isolates, the MIC of nalidixic acid was >256 mg/L and that of ciprofloxacin was >8 mg/L. Corresponding antibiotic resistance genes were detected using PCR, revealing that resistance to sulphonamides was connected mainly with the gene *sul1* (16 isolates). Only one isolate contained the gene *sul2*, and the combination of *sul1* and *sul2* was found in four isolates. Resistance to tetracycline was connected with the presence of the gene *tet*(A). Most ESBL-producing isolates also contained $bla_{\text{OXA-1}}$ and the *aac(6′)-Ib-cr* aminoglycoside–quinolone resistance gene.

Class 1 and class 2 integrons were detected in 23 ESBL-producing *E. coli*. Class 1 integrons 1.7 kb in size with *dfr17-aadA5* gene cassettes were the most common, being found in 19 isolates. Two isolates contained a 2.5 kb integron with *dfr17-aadA5* or an integron 1 kb in size containing the *aadA1* gene cassette. Class 2 integrons 2.5 kb in size and containing a *dfr1-sat-aadA1* gene cassette were detected in two of the isolates.

Phylogenetic grouping and O typing

Twenty of the CTX-M-producing isolates belonged to phylogenetic group B2. Isolates of A (four isolates) and D (two) phylogenetic groups were also detected. Nineteen isolates of the B2 group were typed as O25 by serotyping. Allele-specific PCR confirmed the presence of the *rfbO25b* locus in these isolates, showing that all of them belonged to the O25b type. Another four isolates belonged to the O55 (one), O101 (two) and O153 (one) serotypes and three isolates were not typeable by standard agglutination tests.

PFGE analysis and MLST

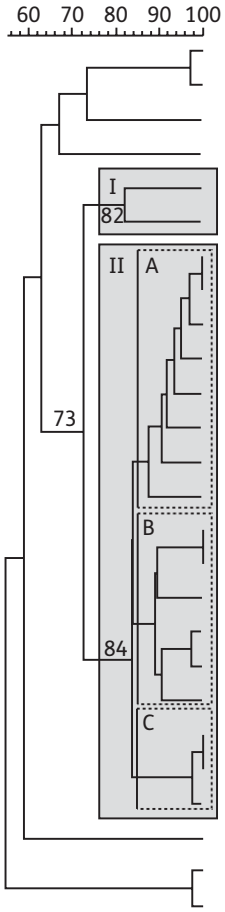
Cluster analysis of macrorestriction patterns was performed on all 26 ESBL-producing *E. coli* isolates. Nineteen CTX-M-15-producing B2-O25b *E. coli* isolates revealed two main clusters, I and II, at a genetic linkage of 73% (Figure 1). Cluster I consisted of just two isolates and both of them differed from cluster II in the absence of the *afa/dra* operon and different constitutions of the upstream region of $bla_{\text{CTX-M-15}}$. Isolates belonging to cluster II shared 84% banding pattern similarity. Three PFGE groups, A–C, were found inside cluster II. Group A comprised eight isolates with genetic linkage of 88%, group B comprised six isolates with 89% band similarity, and group C comprised three isolates with 98% band similarity. All 17 B2-O25b *E. coli* isolates from cluster II were positive for the *afa/dra* operon and contained an integron 1.7 kb in size with *dfr17-aadA5* gene cassettes, and the IS*26* element was found upstream of $bla_{\text{CTX-M-15}}$. The isolates from groups A and B both carried *aac(6′)-Ib-cr* and $bla_{\text{OXA-1}}$ genes, whereas isolates from group C were negative for these genes. MLST analysis in all isolates from cluster I and five selected isolates from cluster II showed that they belonged to the epidemiologically important clone ST131.

Plasmids associated with $bla_{\text{CTX-M}}$ in E. coli

From a total of 26 CTX-M-producing isolates, conjugation to *E. coli* and/or *Salmonella* was demonstrated only in 4. Transconjugants to *E. coli* and *Salmonella* were obtained from one ST131 strain (OV54/B). In this strain, the gene $bla_{\text{CTX-M-15}}$ was located on a 140 kb IncFIA conjugative plasmid together with *aac(6′)-Ib-cr*, $bla_{\text{OXA-1}}$ and *tet*(A) genes. Another transconjugant was obtained from a B2-O101 isolate (OV86/B), where the gene $bla_{\text{CTX-M-15}}$ was harboured by a 120 kb FIB plasmid along with *aac(6′)-Ib-cr* and $bla_{\text{OXA-1}}$ genes. The two CTX-M-1-positive isolates showing related PFGE profiles (OV9/B, OV10/B) conjugated to *E. coli* and *Salmonella*, and 40 kb IncN plasmids of identical HincII profiles were found in these isolates. No transconjugants were obtained from CTX-M-15-producing B2-O25b-ST131 isolates of cluster II. Chemical transformation of B2-O25b-ST131 isolates was successful only in isolate OV22/B from cluster II, where a 95 kb plasmid of IncFIA with $bla_{\text{CTX-M-15}}$, *aac(6′)-Ib-cr*, $bla_{\text{OXA-1}}$ and *tet*(A) genes was found. This OV22/B transformant showed an IncF plasmid EcoRV profile related to the OV54/B transconjugant.

ESBL-producing Klebsiella spp.

Four ESBL-producing *K. pneumoniae* and one *K. oxytoca* were isolated (Table 1). All isolates were multiresistant and produced CTX-M-15, and IS*Ecp1* was identified upstream of $bla_{\text{CTX-M-15}}$. The XbaI PFGE profile of each *K. pneumoniae* isolate was unique except that two isolates, OV17/B and OV28/B, showed only one band difference. Using MLST, *K. pneumoniae* isolates were identified as ST14 (one isolate), ST321 (two isolates) and ST323 (one isolate). Conjugative transfer of ESBL plasmids to



Strain no.	Sampl. day	PG	O type	CTX-M	Upstream of $bla_{CTX-M-15}$[a]			OXA-1	*aac(6′)-Ib-cr*	*afa/dra*	Replicon	Integron[b]	Additional antibiotic resistance		MIC (mg/L)	
					IS*Ecp1* 5′	IS*Ecp1* UP	*tnpA* IS*26*						phenotype	genes	NLA	CIP
OV88/B	34	A	O101	15	2.5	0.25	–	+	+	–	FIB	–	GEN, STR, SUL	*strA, sul2*	>256	>8
OV86/B	31	A	O101	15	2.5	0.25	–	+	+	–	FIA, FIB	Ic	GEN, STR, SUL	*strA, sul1, sul2*	>256	>8
OV59/B	17	D	NT	15	2.5	0.25	–	–	–	–	Y	I2	–	–	128	>8
OV25/B	8	D	O153	15	2.5	0.25	–	+	–	–	–	I2	STR, SUL, SXT, TET	bla_{TEM-1}, *strA, tet*(D), *cat*	256	4
OV54/B	16	B2	O25b	15	2.5	0.25	–	+	+	–	FIA	Ia	TET	*tet*(A), *sul1*	>256	>8
OV32/B	10	B2	O25b	15	–	0.25	0.9	+	+	–	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1, sul2*	>256	>8
OV45/B	12	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	STR, SUL, SXT, TET	*strA, tet*(A), *sul1, sul2*	>256	>8
OV46/B	12	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV2/B	2	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	STR, SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV31/B	10	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV24/B	8	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	bla_{TEM-1b}, *tet*(A), *sul1*	>256	>8
OV4/B	2	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	STR, SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV5/B	2	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT	*sul1, sul2*	>256	>8
OV22/B	7	B2	O25b	15	–	–	0.4	+	+	+	FIA, Y	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV68/B	26	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV70/B	28	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV56CH	13	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV3/B	2	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	bla_{TEM-1b}, *tet*(A), *sul1*	>256	>8
OV8/B	3	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	bla_{TEM-1b}, *tet*(A), *sul1*	>256	>8
OV95/B	38	B2	O25b	15	–	–	0.4	+	+	+	FIA	Ia	SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV10/B	3	B2	O25b	15	–	–	0.4	–	–	+	FIA	Ia	STR, SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV19/B	4	B2	O25b	15	–	–	0.4	–	–	+	FIA	Ia	STR, SUL, SXT, TET	*tet*(A), *sul1*	>256	>8
OV9/B	3	B2	O25b	15	–	–	0.4	–	–	+	FIA	Ia	SUL, SXT	*sul1*	>256	>8
OV27/B	9	B2	O55	15	–	–	0.4	–	+	–	P, I1	Ib	TET	*tet*(A)	>256	0.25
OV18/B	5	A	NT	1	–	0.3	0.6	–	–	–	N	–	–	–	256	>8
OV20/B	6	A	NT	1	–	0.3	0.6	–	–	–	N	–	–	–	256	>8

Figure 1. Characterization of 26 ESBL-producing *E. coli* isolates from a WWTP. The dendrogram consists of two main PFGE clusters, I and II, with genetic relatedness of 73%; cluster II contains three groups of isolates, named A, B and C, with 84% band similarity. PG, phylogenetic group; O type, serotype of *E. coli* determined by conventional serotyping and/or PCR; NT, not typeable; GEN, gentamicin; STR, streptomycin; SUL, sulphonamides; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin. [a]IS*Ecp1* 5′, linkage between the 5′ region of IS*Ecp1* and bla_{CTX-M}; IS*Ecp1* UP, linkage between IS*Ecp1* and bla_{CTX-M} using forward primer for transposase gene of IS*Ecp1* and reverse primer for bla_{CTX-M}; *tnpA* IS*26*, linkage between IS*26* and bla_{CTX-M}. [b]Ia, class 1 integron 1.7 kb: *dfr17-aadA5*; Ib, class 1 integron 1 kb: *aadA1*; Ic, class 1 integron 2.5 kb: *dfr17-aadA5*; I2, class 2 integron 2.5 kb: *dfr1-sat-aadA1*.

Table 1. CTX-M-15-carrying plasmids from *E. coli* and *Klebsiella* isolates from a WWTP

	Donor strain					Plasmid			
Strain no.	species	PG	O type	MLST	CTX-M	Inc	RFLP	size (kb)	additional antibiotic resistance genes
OV18/B.tc	*E. coli*	A	NT	ND	1	N	I	40	—
OV20/B.tc	*E. coli*	A	NT	ND	1	N	I	40	—
OV22/B.tf	*E. coli*	B2	O25b	ST131	15	FIA	IIa	95	bla_{OXA-1}, *aac(6′)-Ib-cr*, *tet*(A)
OV54/B.tc	*E. coli*	B2	O25b	ST131	15	FIA	IIb	140	bla_{OXA-1}, *aac(6′)-Ib-cr*, *tet*(A)
OV86/B.tc	*E. coli*	B2	O101	ND	15	FIB	III	120	bla_{OXA-1}, *aac(6′)-Ib-cr*
OV17/B.tf	*K. pneumoniae*	ND	ND	ST321	15	FIIK	V	110	bla_{TEM-1}, bla_{OXA-1}, *strA*, *tet*(A), *aac(6′)-Ib-cr*, *aac(3′)-II*, *qnrB1*
OV28/B.tf	*K. pneumoniae*	ND	ND	ST321	15	FIIK	V	110	bla_{TEM-1}, bla_{OXA-1}, *strA*, *tet*(A), *aac(6′)-Ib-cr*, *aac(3′)-II*, *qnrB1*
OV60/B.tf	*K. pneumoniae*	ND	ND	ST323	15	FIIK	V	110	bla_{TEM-1}, bla_{OXA-1}, *strA*, *tet*(A), *aac(6′)-Ib-cr*, *aac(3′)-II*, *qnrB1*
OV88/B.tc	*K. oxytoca*	ND	ND	ND	15	FIA/FIB	IV	130	—

tc, transconjugant; tf, transformant; PG, phylogenetic group; O type, serotype of *E. coli* determined by conventional serotyping and/or PCR; NT, not typeable; ND, not determined; Inc, plasmid incompatibility group; RFLP, restriction fragment length polymorphism profile of plasmid DNA using EcoRV or HincII.

E. coli and/or *Salmonella* was demonstrated. Characterization of ESBL-carrying plasmids showed the presence of $bla_{CTX-M-15}$ on a 110 kb IncFIIK conjugative plasmid in the *K. pneumoniae* isolates. Multiple antibiotic resistance genes [bla_{TEM-1}, bla_{OXA-1}, *strA*, *tet*(A), *aac(6′)-Ib-cr*, *aac(3′)-II* and *qnrB1*] were located on this FIIK plasmid together with $bla_{CTX-M-15}$. FIIK plasmids showed related EcoRV profiles. The nucleotide sequence of the *copA* gene of all FIIK plasmids was identical and showed a novel allele. The sequence of the new *copA* gene allele differed from those of alleles K3 (AJ009980) and K5 (FJ628167) (available at http://pubmlst.org/plasmid/) at one and two nucleotides, respectively, and has been assigned to the allele K6. In the transformant of the *K. oxytoca* isolate, OV88/B, the gene $bla_{CTX-M-15}$ was found on a 130 kb conjugative plasmid of the FIA/FIB incompatibility group. No other antibiotic resistance genes were found on this plasmid.

Upstream region of bla_{CTX-M} genes

PCR using different primer combinations identified the insertion sequence IS*Ecp1*, entirely or partially truncated, upstream of the bla_{CTX-M} gene in eight isolates (Figure 1). PCR using the IS*Ecp1* 5′ primer showed a 2.5 kb fragment of the entire IS*Ecp1* in four non-ST131 *E. coli* isolates and one ST131 (OV54/B) *E. coli* isolate as well as in all *Klebsiella* isolates. The upstream region of $bla_{CTX-M-15}$ in all ST131 isolates from cluster II, analysed by PCR, contained the transposase gene of the insertion sequence IS*26*. However, PCR with the IS*Ecp1* 5′ primer as well as a primer binding to the *tnpA* gene of IS*Ecp1* were negative. As confirmed by sequencing, these strains had IS*26* flanking a partially truncated IS*Ecp1*, thus separating the $bla_{CXT-M-15}$ from its usual promoter. Two CTX-M-1-positive isolates (OV18/B, OV20/B) and one ST131 isolate (OV32/B) also showed the presence of IS*26* disrupted by IS*Ecp1* at a different position compared with isolates from cluster II and not separating $bla_{CTX-M-15}$ from its promoter. Both CTX-M-1-positive isolates had a 48 bp region (W sequence) upstream of the $bla_{CTX-M-1}$ gene and an additional 32 bp X sequence, as observed in a previous sequence reported in the GenBank database (accession no. AM003904). The CTX-M-15-positive *E. coli* and *Klebsiella* isolates were characterized by the presence of only the 48 bp W sequence, as previously described (AM040707).

Discussion

Human sewage represents an important source of pathogenic and antibiotic-resistant bacteria and of antibiotic residues in the environment. We documented the frequent presence of bacteria of the Enterobacteriaceae with emerging resistance mechanisms in treated water from a municipal WWTP. There are only a few reports on the isolation of ESBL-producing bacteria from wastewater ecosystems.[26]

The presence of low concentrations of antibiotics in WWTPs has been documented,[27] and these antibiotics may exert selective pressure favouring resistant strains. Cephalosporins and fluoroquinolones hold the third and fourth positions on the European antibiotics market and their use is tending to increase.[28] They are extracted into the urine and discharged into hospital and municipal wastewater.[29] Fluoroquinolones, trimethoprim and sulphonamides have been found to be poorly removed during wastewater treatment[30,31] and therefore might exert selective pressure on bacteria within the sewage plant.

Using sequencing and MLST analysis, we found that 73% of CTX-M-15, phylogroup B2 *E. coli* strains corresponded to the worldwide emerging O25b-ST131 clonal group. This clone was detected in almost 25% of treated wastewater samples during the 45 day sampling period. Recently, ST131, O25:H4 and phylogenetic group B2 were shown to characterize the major clone (88% of total) among CTX-M-15-producing *E. coli* isolates in Europe, Asia and North America.[32] To our knowledge, this is the first evidence of this emerging multiresistant clone in municipal wastewater. The multiresistant CTX-M-producing ST131 clone has recently been isolated from river water in the UK,[33] probably resulting from significant influx of raw sewage contamination. This provides evidence of the survival of the ST131 clone in river water and its potential further dissemination into the environment and wildlife.

Association of IS*26* and $bla_{CTX-M-15}$ in the ST131 clone has been observed previously.[34] The *afa/dra* operon is a virulence

gene encoding Dr family adhesins found in diffusely adhering *E. coli* strains, including CTX-M-15-producing O25b-ST131 clones.[35] In our study, IS*26* linked to the gene $bla_{CTX\text{-}M\text{-}15}$ as well as the *afa/dra* operon were found in all the ST131 strains from the dominant cluster II. CTX-M-15-producing O25b-ST131 *E. coli* clones with the *afa/dra* operon and IS*26* element flanking $bla_{CTX\text{-}M\text{-}15}$ have been isolated from human urinary tract infections in Spain.[34] Interestingly, *E. coli* isolates from cluster II/PFGE group A exhibited PFGE patterns closely related (having one- and two-band differences) to a human B2-O25b-ST131 CTX-M-15-producing, *afa/dra* operon-positive strain, DSM22664, representing the human pandemic virulent group of clonally related strains isolated from a case of bloody enteritis in Germany.[32,35] It seems that this diffusely adhering ST131 clone with IS*26* flanking $bla_{CTX\text{-}M\text{-}15}$ is successfully spreading in the human population as well as in companion animals in some European countries.[35]

Interestingly, all ST131 *E. coli* isolates in our study harboured an FIA plasmid. Other studies also show that the $bla_{CTX\text{-}M\text{-}15}$ in ST131 clones is not only located on FII plasmids but also on plasmids of the FI incompatibility group.[35,36] It has been demonstrated that a temperature of 20°C is more conducive than 37°C (the temperature used in our study) to the transfer of resistance plasmids between *E. coli* strains contained in river water in the laboratory,[37] which may explain the low level of conjugative transfer of FIA plasmids in ST131 clones observed in our study. Nevertheless, plasmid analysis of one transconjugant and one transformant of two ST131 strains showed the presence of $bla_{CTX\text{-}M\text{-}15}$ on multiresistance plasmids along with $bla_{OXA\text{-}1}$, aminoglycoside–fluoroquinolone gene *aac(6′)-Ib-cr* and the tetracycline resistance determinant *tet*(A). Association of $bla_{CTX\text{-}M\text{-}15}$ with these antibiotic resistance genes present on IncF plasmids is well documented.[6] Both the CTX-M-1-positive isolates from WWTP harboured the $bla_{CTX\text{-}M\text{-}1}$ gene on a conjugative plasmid of incompatibility group N. It is noteworthy that IncN plasmids with related restriction fragment length polymorphism profiles of plasmid DNA have been isolated from CTX-M-1-producing animal and human *E. coli* strains in the Czech Republic.[11,38,39]

In our study, four CTX-M-15-producing *K. pneumoniae* strains of three different MLST STs (ST14, ST321 and ST323) were isolated from treated wastewater and all the isolates harboured large FIIK plasmids with multiple antibiotic resistance genes and conjugative ability to *E. coli*. These FIIK plasmids identified in *Klebsiella* are mostly regarded as virulence plasmids.[25] Association of $bla_{CTX\text{-}M\text{-}15}$ with IncFIIK has recently been documented and a high capacity for these plasmids to diffuse and persist over time has been suggested.[40] IncFII plasmids harbouring the CTX-M-15 gene and associated with multiple resistance genes [$bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$, *tet*(A), *aac(6′)-Ib-cr* and *aac(3)-II*], as found in *Klebsiella* isolates in our study, have been found previously also in mainly human *E. coli* and *K. pneumoniae* isolates from different continents, thus demonstrating their worldwide dissemination.[41,42] To our knowledge, this is the first study documenting these multiple antibiotic resistance elements on FIIK plasmids. The differences in the presence of particular antibiotic resistance genes in our plasmids compared with other studies suggest new genetic rearrangements. The results highlight the evolution of IncF plasmids into new variants containing novel antibiotic resistance elements. Further studies are needed to examine the dissemination of FIIK multiple resistance plasmids and their role in spreading ESBL and quinolone resistance genes in Enterobacteriaceae in the Czech Republic and elsewhere.

This study demonstrated the insufficient effect of the treatment process in removing bacteria harbouring antibiotic resistance, increased survival of specific multiresistant clones, such as CTX-M-15-producing *E. coli* ST131, and the risk of transmitting clinically important antibiotic-resistant strains into the environment through municipal wastewater. Further studies are needed to understand the dissemination of ST131 pandemic clones in water ecosystems and their impact on human health.

Acknowledgements

We would like to thank Lucie Novakova, Simona Krepelova, Petra Slamova, Daniela Kohutova, Eva Suchanova and Marie Slavikova for excellent laboratory cooperation. Our thanks go to Pavel Alexa (Veterinary Research Institute, Brno, Czech Republic) for serotyping of *E. coli*, to Henrik Hasman and Lina Cavaco (National Food Institute, Copenhagen, Denmark) and Alessandra Carattoli (Instituto Superior di Sanita, Rome, Italy) for positive control strains, to Pavel Svec (Czech Collection of Microorganisms, Brno, Czech Republic) for help with PFGE analysis and to Jan Kyzlink from the WWTP in Brno, Czech Republic. This publication made use of the Plasmid MLST web site (http://pubmlst.org/plasmid/) developed by Keith Jolley and sited at the University of Oxford.

Funding

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (MSM6215712402), the Czech Science Foundation (P502/10/P083) and the project 'CEITEC - Central European Institute of Technology' (CZ.1.05/1.1.00/02.0068) of the European Regional Development Fund.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Zhang X, Zhang T, Fang HHP. Antibiotic resistance genes in water environment. *Appl Microbiol Biotechnol* 2009; **82**: 397–414.

2 Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M *et al*. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol* 2007; **60**: 166–76.

3 Andersen SR. Effects of waste water treatment on the species composition and antibiotic resistance of coliform bacteria. *Curr Microbiol* 1993; **26**: 97–103.

4 Guenther S, Grobbel M, Beutlich J *et al*. Detection of pandemic B2-O25-ST131 *Escherichia coli* harbouring the CTX-M-9 extended-spectrum β-lactamase type in a feral urban brown rat (*Rattus norvegicus*). *J Antimicrob Chemother* 2010; **65**: 582–4.

5 Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *J Antimicrob Chemother* 2011; **66**: 1–14.

6 Woodford N, Carattoli A, Karisik E *et al*. Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrob Agents Chemother* 2009; **53**: 4472–82.

7 Naseer U, Sundsfjord A. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. *Microb Drug Resist* 2011; **17**: 83–97.

8 Moore B. The detection of paratyphoid carriers in towns by means of sewage examination. *Mon Bull Minist Health Public Health Lab Serv* 1948; **9**: 72–8.

9 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Eighteenth Informational Supplement M100-S18*. CLSI, Wayne, PA, USA, 2008.

10 Literak I, Dolejska M, Janoszowska D *et al*. Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum β-lactamase and QnrS, in waterbirds on the Baltic Sea coast of Poland. *Appl Environ Microbiol* 2010; **76**: 8126–34.

11 Dolejska M, Duskova E, Rybarikova J *et al*. Plasmids carrying $bla_{CTX-M-1}$ and *qnr* genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. *J Antimicrob Chemother* 2011; **66**: 757–64.

12 Eckert C, Gautier V, Arlet G. DNA sequence analysis of the genetic environment of various bla_{CTX-M} genes. *J Antimicrob Chemother* 2006; **57**: 14–23.

13 Eckert C, Gautier V, Saladin-Allard M *et al*. Dissemination of CTX-M-type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. *Antimicrob Agents Chemother* 2004; **48**: 1249–55.

14 Diestra K, Juan C, Curiao T *et al*. Characterization of plasmids encoding bla_{ESBL} and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2009; **63**: 60–6.

15 Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 2000; **66**: 4555–8.

16 Salajka E, Salajkova Z, Alexa P *et al*. Colonization factor different from K88, K99, F41 and 987P in enterotoxigenic *Escherichia coli* strains isolated from postweaning diarrhea in pigs. *Vet Microbiol* 1992; **32**: 163–75.

17 Clermont O, Dhanji H, Upton M *et al*. Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. *J Antimicrob Chemother* 2009; **64**: 274–7.

18 Carattoli A, Bertini A, Villa L *et al*. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219–28.

19 *One-Day (24–28 h) Standardized Laboratory Protocol for Molecular Subtyping of Escherichia coli O157:H7, non-typhoidal Salmonella serotypes, and Shigella sonnei by Pulsed Field Gel Electrophoresis (PFGE)*. http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf (1 May 2008, date last accessed).

20 Wirth T, Falush D, Lan R *et al*. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 2006; **60**: 1136–51.

21 Diancourt L, Passet V, Verhoef J *et al*. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005; **43**: 4178–82.

22 Olesen I, Hasman H, Aarestrup FM. Prevalence of β-lactamases among ampicillin-resistant *Escherichia coli* and *Salmonella* isolated from food animals in Denmark. *Microb Drug Resist* 2004; **10**: 334–40.

23 Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979; **7**: 1513–23.

24 Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem* 1995; **226**: 235–40.

25 Villa L, Garcia-Fernandez A, Fortini D *et al*. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. *J Antimicrob Chemother* 2010; **65**: 2518–29.

26 Reinthaler FF, Feierl G, Galler H *et al*. ESBL-producing *E. coli* in Austrian sewage sludge. *Water Res* 2010; **44**: 1981–5.

27 Watkinson AJ, Micalizzi GB, Graham GM *et al*. Antibiotic-resistant *Escherichia coli* in wastewaters, surface waters, and oysters from an urban riverine system. *Appl Environ Microbiol* 2007; **73**: 5667–70.

28 Sande-Bruinsma N, Grundmann H, Verloo D *et al*. Antimicrobial drug use and resistance in Europe. *Emerg Infect Dis* 2008; **14**: 1722–30.

29 Lindberg R, Jarnheimer P, Olsen B *et al*. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosphere* 2004; **57**: 1479–88.

30 Gobel A, Thomsen A, McArdell CS *et al*. Extraction and determination of sulfonamides, macrolides, and trimethoprim in sewage sludge. *J Chromatogr A* 2005; **1085**: 179–89.

31 Golet EM, Strehler A, Alder AC *et al*. Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal Chem* 2002; **74**: 5455–62.

32 Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V *et al*. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. *J Antimicrob Chemother* 2008; **61**: 273–81.

33 Dhanji H, Murphy NM, Akhigbe C *et al*. Isolation of fluoroquinolone-resistant O25b:H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum β-lactamase from UK river water. *J Antimicrob Chemother* 2011; **66**: 512–6.

34 Oteo J, Orden B, Bautista V *et al*. CTX-M-15-producing urinary *Escherichia coli* O25b-ST131-phylogroup B2 has acquired resistance to fosfomycin. *J Antimicrob Chemother* 2009; **64**: 712–7.

35 Ewers C, Grobbel M, Stamm I *et al*. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-β-lactamase-producing *Escherichia coli* among companion animals. *J Antimicrob Chemother* 2010; **65**: 651–60.

36 Gonullu N, Aktas Z, Kayacan CB *et al*. Dissemination of CTX-M-15 β-lactamase genes carried on Inc FI and FII plasmids among clinical isolates of *Escherichia coli* in a university hospital in Istanbul, Turkey. *J Clin Microbiol* 2008; **46**: 1110–2.

37 Grabow WO, Prozesky OW. Drug resistance of coliform bacteria in hospital and city sewage. *Antimicrob Agents Chemother* 1973; **3**: 175–80.

38 Dolejska M, Jurcickova Z, Literak I *et al*. IncN plasmids carrying $bla_{CTX-M-1}$ in *Escherichia coli* isolates on a dairy farm. *Vet Microbiol* 2011; **149**: 513–6.

39 Literak I, Petro R, Dolejska M *et al*. Antimicrobial resistance in fecal *Escherichia coli* isolates from healthy urban children of two age groups in relation to their antibiotic therapy. *Antimicrob Agents Chemother* 2011; **55**: 3005–7.

40 Coelho A, González-Lopez JJ, Miro E *et al*. Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. *Int J Antimicrob Agents* 2010; **36**: 73–8.

41 Ruiz E, Rojo-Bezares B, Sáenz Y *et al*. Outbreak caused by a multi-resistant *Klebsiella pneumoniae* strain of new sequence type ST341 carrying new genetic environments of *aac(6′)-Ib-cr* and *qnrS1* genes in a neonatal intensive care unit in Spain. *Int J Med Microbiol* 2010; **300**: 464–9.

42 Karisik E, Ellington MJ, Pike R *et al*. Molecular characterization of plasmids encoding CTX-M-15 β-lactamases from *Escherichia coli* strains in the United Kingdom. *J Antimicrob Chemother* 2006; **58**: 665–8.