Extended-spectrum β -lactamase producing *Escherichia coli* in hospital wastewaters and sewage treatment plants in Queensland, Australia

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Abstract: We investigated the prevalence of extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* in untreated hospital wastewaters and 2 sewage treatment plants (STPs). A collection of 252 ESBL-producing *E. coli* isolates from hospital wastewater and STPs were typed and tested for resistance to 17 antimicrobial agents and for the presence of integron-associated integrases (*intI* gene) and ESBL genes. Eighty-nine percent ($n = 176$) of the ESBL-producing *E. coli* strains from hospital wastewater were found in more than 1 sample (common types), with 1 common type accounting for 35% of isolates, found in all samples. These strains were also resistant to up to 9 non- β -lactam antibiotics and showed the same pattern of resistance in all samples. More than 73% of the hospital wastewater isolates possessed SHV-type ESBL as opposed to isolates from STPs that carried only CTX-M-type ESBL genes. The prevalence of the *intI* gene did not differ between the sources of the isolates. Certain ESBL-producing *E. coli* were dominant in hospital wastewaters. These strains possessed β -lactamase genes that were different from isolates found in STPs. From a public health point of view, the presence of such a high level of ESBL-producing *E. coli* strains in hospital wastewaters is of great importance.

Key words: *Escherichia coli*, ESBL, hospital wastewater, sewage treatment plant, antibiotic-resistant bacteria.

Résumé : Nous avons enquêté sur la prévalence d'*Escherichia coli* producteurs de β -lactamase à spectre étendu (BLSE) dans les eaux usées non traitées d'un hôpital et dans deux usines d'épuration. À partir d'une banque de 252 isolats d'*E. coli* producteurs de BLSE provenant d'eaux d'hôpitaux et d'usines d'épuration, on a procédé au typage, à des tests de résistance à 17 antimicrobiens, et à l'analyse de la présence d'intégrases associées à un intégron (gène *intI*) et de gènes de BLSE. Quatre-vingt-neuf pour cent ($n = 176$) des souches d'*E. coli* productrices de BLSE issues d'eaux usées d'hôpital se retrouvaient dans plus d'un échantillon (types communs). Un type commun représentait 35 % de tous les isolats et était présent dans tous les échantillons. En outre, ces souches étaient résistantes à jusqu'à 9 antibiotiques non β -lactamines; le même profil de résistance s'observait dans tous les échantillons. Plus de 73 % des isolats d'eaux usées d'hôpital renfermaient un BLSE de type SHV, contrairement aux isolats d'usines d'épuration qui étaient uniquement porteurs de gènes BLSE de type CTX-M. La prévalence du gène *intI* ne variait pas selon la source. Certains *E. coli* producteurs de BLSE étaient dominants dans les eaux usées d'hôpital. Ces souches renfermaient des gènes de β -lactamase différents de ceux d'isolats d'usines d'épuration. Du point de vue de la santé publique, on considère que la présence d'un taux si élevé de souches d'*E. coli* productrices de BLSE dans des eaux usées d'hôpital est digne de mention. [Traduit par la Rédaction]

Mots-clés : *Escherichia coli*, BLSE, eaux usées d'hôpital, usine d'épuration, bactérie antibiorésistante.

Introduction

The wide application of antimicrobial agents in clinical settings, veterinary medicine, livestock industries, and aquaculture has led to large-scale dissemination of antibiotic-resistant bacteria in many environments (Kolar et al. 2001; Gaskins et al. 2002; Mölsted et al. 2002; Kummerer 2009a). These resistant strains generally reach the environment from uncontrolled discharges of urban wastewater (Kim and Aga 2007), including hospital wastewater (Chitnis et al. 2004), and animal husbandry via manure (Goni-Urriza et al. 2000). In addition, various wastewaters contaminated with unmetabolized and (or) unused antibiotics may also pose a selective pressure on bacteria, leading to the development

of antibiotic-resistant strains in the environment (Kummerer 2004). Resistance genes can then be transferred between bacteria via mobile genetic elements (Droge et al. 1998). One of the mobile element integrons that mediate integration of resistance genes may also be involved (Partridge et al. 2009) resulting in the development of multidrug-resistant bacteria. Most studies surveying antimicrobial resistance have focused on clinical isolates, while the survival and prevalence of antibiotic-resistant bacteria in the environment has not been well investigated. Furthermore, while hospitals have been regarded as a major source of dissemination of antibiotic-resistant bacteria, very little is known about the contribution of other sources in the spread of these strains. For instance, it has been shown that the human intestinal flora of

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healthy individuals may serve as a reservoir for multidrug-resistant bacteria (Valverde et al. 2004; Mesa et al. 2006). These bacteria can be released into the receiving sewer system and end up in sewage treatment plants (STPs) (Kummerer 2004).

Escherichia coli, a normal inhabitant of the human gut, is one of the most commonly found bacteria in community STPs. Some of these *E. coli* strains may also receive or carry multidrug-resistant genes during their transition in sewer systems or presence in STPs. Therefore analysis of these bacteria in hospital wastewaters and STPs can provide a better understanding of the prevalence and spread of antibiotic-resistant strains in the community.

There are growing concerns about resistant pathogenic *E. coli* (Kaper et al. 2004; Pitout 2012) and their ecotoxic effects in the environment (Gonzalez-Candelas et al. 2011). Certain strains have been shown to carry genes coding for enzymes that hydrolyze a wide range of β -lactam antibiotics and are referred to as extended-spectrum β -lactamase (ESBL)-producing strains. Recent studies have shown that the prevalence of ESBL-producing *E. coli* in different countries ranges between 1.3% and 42.2%, with the highest rate belonging to *E. coli* isolated from intra-abdominal body sites of hospitalized patients (Nijssen et al. 2004; Valverde et al. 2004; Hirakata et al. 2005; Hawser et al. 2009).

In view of the significant role of pathogenic *E. coli* strains in human diseases and their prevalence in wastewaters, this study was undertaken to search for the prevalence of ESBL-producing *E. coli* in hospital wastewater and STPs. A special reference was made to determining the prevalence of different classes of ESBL genes as well as the presence of difference classes of integrons among these isolates.

Materials and methods

Sites and sampling

Untreated wastewater samples were collected for 14 consecutive weeks (once a week) from the main outlet pipe of a metropolitan hospital in South East Queensland between March and June 2011. During the same period, 4 samples were collected from the incoming pretreatment wastewater of 2 STPs (4 samples each) over 4 weeks. Both STPs service an equivalent population of 130 000 and they both have a 12- to 13-day sludge-treatment cycle. Besides, none of them received samples from the hospital included in this study and as such could serve as a source of community strains of ESBL-producing *E. coli*. All samples were collected between 0730 and 0830 on the same day of the week and were processed in accordance with the Australian and New Zealand Standards for Water Microbiology and Water Quality Sampling (Australian and New Zealand Standards 1998a, 1998b, 2007). In brief, wastewaters were collected in 500 mL sterile microbiological containers mounted onto a handle of appropriate length using a "grab-sampling" technique. They were transported to the laboratory on ice and processed within 4 h of collection.

Isolation and identification of ESBL-producing *E. coli*

Wastewater samples were processed using serial dilutions and cultured on 2 mFC agar plates (Oxoid) containing 32 μ g/mL ceftazidime and 16 μ g/mL aztreonam. After an overnight incubation at 44 °C, up to 24 suspected *E. coli* colonies (where possible) were randomly picked up from each sample and tested for the presence of the highly specific *E. coli* universal stress protein (*uspA*) gene. Chromosomal DNA was extracted using a Genomic DNA Extraction kit (Blood/Bacterial/Cultured Cells) (Real Biotech Corporation), and the *uspA* gene was amplified as described by Chen and Griffiths (1998). PCR products were electrophoresed on a 2% agarose gel (AMRESCO, Astral Scientific) in 0.6 $\times$ TBE (Tris-borate-EDTA), stained with ethidium bromide, and viewed under ultraviolet light with a Gel Doc system (BioRad, Australia). DNA were then maintained in -20 °C in elution buffer for further use, and the

strains were stored on tryptone soy broth with 15% glycerol at -80 °C for further analysis.

Phenotypic confirmation of ESBL producers

The confirmation of ESBL production of the isolates was done according to the method described by Bell et al. (2006). The synergy between the β -lactam antibiotic disk and clavulanate was monitored by placing a disk of β -lactamase inhibitor - β -lactam antibiotic combination drug and a disk of β -lactam antibiotic on an inoculated Mueller-Hinton agar plate with 25 mm apart (center to center). These included amoxicillin-clavulanic acid (60 μ g), ceftazidime (10 μ g), cefotaxime (5 μ g), and aztreonam (30 μ g). The presence of a clear extension of the edge of the β -lactam antibiotic inhibition zone toward the disk containing β -lactamase inhibitor indicated ESBL production. If resistance zones were small, making it difficult to identify a clear zone between disks, the test was repeated, placing the antibiotic disks 20 and 15 mm apart (center to center). Two ESBL-producing *E. coli* strains (K6 and EC10) (Gündoğdu et al. 2012) and a susceptible *E. coli* strain ACM5185 were included in all tests as the positive and negative controls, respectively. In all, 252 ESBL-producing *E. coli* were isolated from hospital wastewaters ($n = 198$) and STPs ($n = 54$).

Typing of isolates

All phenotypically confirmed ESBL-producing *E. coli* isolates (i.e., 252 isolates) were typed using multilocus variable number tandem repeat analysis (MLVA), PhP fingerprinting, and phylogenetic grouping. MLVA was performed as described previously by Lindstedt et al. (2007), targeting 7 tandem repeats (CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, and CVN015), using 2 multiplexes and 1 uniplex PCR. However, the fluorescent labels used for the forward primers were changed as follows: CVN001-VIC, CVN002-VIC, CVN003-NED, CVN004-NED, CVN007-FAM, CVN014-FAM, and CVN015-PET. PCR products were sized on a 16-capillary Applied Biosystems 3130xl Genetic Analyzer with a 50 cm capillary, and POP-7 was used as the separation matrix. Fragment sizing was performed using Gene Mapper version 4.0 software (Applied Biosystems) using LIZ600 as the size standard. Each peak was identified according to fluorescent label and size, and the allele number was assigned based on fragment sizes as described by Lindstedt et al. (2007). Alleles for which amplicons were absent were designated an allele number of "0".

PhP fingerprinting was done using a high-resolution biochemical fingerprinting plate, i.e., PhP-RE plate (PhPlate AB, Stockholm, Sweden), which has been specifically developed for typing of *E. coli* strains (Landgren et al. 2005). Inoculation of the plates was done according to the manufacturer's instruction, and the plates were incubated at 37 °C. The rate of each reaction was evaluated by measuring the absorbance value in each well after 7, 24, and 48 h of incubation using a digital scanner (HP Scanjet 4890 scanner). After the final scan, the PhPlate software was used to create absorbance (A_{620}) data from the scanned plate images, and the mean absorbance in each well over the 3 readings was calculated, yielding the biochemical fingerprint for each isolate. The biochemical fingerprints of the isolates were compared pairwise, and the similarity among the isolates was calculated as the correlation (similarity) coefficient and clustered according to the unweighted pair group method with arithmetic averages (Sneath and Sokal 1973). Isolates showing a similarity coefficient to each other above the default identity level (0.975) of software were regarded as identical and assigned to the same biochemical phenotype. All data handling, including calculations of correlations and coefficients as well as clustering, were performed using the PhPlate software version 4002 (PhPlate AB).

Phylogenetic grouping was done for weekly representative strains using multiplex PCR with primers coding for *chuA* and *yjaA* genes and the DNA fragment TspE4.C2 (Table 1), according to Clermont et al. (2000). Strains belonging to the same biochemical

Table 1. Primers used for identification of *Escherichia coli* strains (i.e., *uspA* gene), phylogenetic grouping, and extended-spectrum β -lactamase types.

Primer	Target(s)	Sequence (5' to 3')	Reference
UspA-F	<i>uspA</i>	CCGATACGCTGCCAATCAGT	Chen and Griffiths 1998
UspA-R		ACGCAGACCGTAGGCCAGAT	
ChuA-F	<i>chu</i>	GACGAACCAACGGTCAGGAT	Clermont et al. 2000
ChuA-R		TGCCGCCAGTACCAAAGACA	
YjaA-F	<i>yjaA</i>	TGAAGTGTACAGGAGACGCTG	Clermont et al. 2000
YjaA-R		ATGGAGAATGCGTTCCTCAAC	
TspE4.C2-F	TspE4.C2 ^a	GAGTAATGTGGGGCATTCA	Clermont et al. 2000
TspE4.C2-R		CGCGCCAACAAAGTATTACG	
TEM25	<i>blaTEM</i>	CCAGAAACGCTGGTGAAGT	Gündoğdu et al. 2012
TEM26		TGACTCCCGTCGTGTAGAT	
SHV25	<i>blaSHV</i>	CCTTTATCGGCCTTCACTCA	Gündoğdu et al. 2012
SHV26		CTTAGCGTTGCCAGTGCTC	
OPR1	<i>blaOXA</i>	GTCTTTCGAGTACGGCATTAA	Vahaboglu et al. 1998
OPR2		ATTTTCTTAGCGGCAACTTAC	
CTXM1-F3	<i>blaCTX-M</i> (group I) ^b	GACGATGTCACTGGCTGAGC	Pitout et al. 2004
CTXM1-R2		AGCCGCCGACGCTAATACA	
TOHO1-2 F	<i>blaCTX-M</i> (group II) ^b	GCGACCTGGTTAACTACAATCC	Pitout et al. 2004
TOHO1-1 R		CGGTAGTATTGCCCTTAAGCC	
CTXM825 F	<i>blaCTX-M</i> (group III) ^b	CGCTTTGCCATGTGCAGCACC	Pitout et al. 2004
CTXM825 R		GCTCAGTACGATCGAGCC	
CTXM914 F	<i>blaCTX-M</i> (group IV) ^b	GCTGGAGAAAAGCAGCGGAG	Pitout et al. 2004
CTXM914 R		GTA AGCTGACGCAACGTCTG	
Int1 F	<i>intI1</i>	CAGTGGACATAAGCCTGTTC	Dillon et al. 2005
Int1 R		CCCAGGCATAGACTGTA	
Int2 F	<i>intI2</i>	CACGGATATGCGACAAAAGGT	Dillon et al. 2005
Int2 R		GATGACAACGAGTGACGAAATG	
Int3 F	<i>intI3</i>	GCCTCCGGCAGCGACTTTACG	Dillon et al. 2005
Int3 R		ACGGATCTGCCAAACCTGACT	

^aDNA fragment.^bGroup I includes CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -28, -29, and -30. Group II includes CTX-M-2, -4, -5, -6, -7, -20, and Toho-1. Group III includes CTX-M-8. Group IV includes CTX-M-9, -13, -14, -16, -17, -18, -19, -21, -27, and Toho-2.

phenotypes, with identical MLVA profiles and phylogenetic groups, were considered as members of the same clone and were regarded as common types, and those differing by 1 or 2 of the 3 methods were regarded as single types.

Antimicrobial resistance testing

From each common type, representatives of ESBL-producing strains found in different weeks of sampling were selected and tested against another 13 antimicrobial agents using the disk diffusion assay on Mueller–Hinton agar according to the CDS method (Bell et al. 2006). Antimicrobial agents included were cefepime (10 μ g), cefotetan (30 μ g), cefoxitin (30 μ g), imipenem (0 μ g), gentamicin (10 μ g), amikacin (30 μ g), tetracycline (30 μ g), sulphamethoxazole (100 μ g), ciprofloxacin (5 μ g), norfloxacin (10 μ g), nalidixic acid (30 μ g), nitrofurantoin (300 μ g), and chloramphenicol (30 μ g). The CDS method reports antimicrobial drug susceptibilities as either “susceptible” or “resistant”, and therefore, the results are not expressed as minimal inhibitory concentration values, and were therefore interpreted as susceptible (S) or resistant (R).

Characterization of β -lactamase-encoding genes

Representative strains showing a positive result for ESBLs were further tested for the presence of 4 common ESBL genes encoding TEM-, SHV-, CTX-M-, and OXA-type β -lactamases, using PCR. For CTX-M, 4 different primers were used, referred to as CTX-M1, TOHO1, CTXM825, and CTXM914. The selected primers for detection of all genes is shown in Table 1. The uniplex PCR reactions for genes encoding TEM-, SHV-, and CTX-M-types were carried out in a 30 μ L volume containing 3 μ L of 10 $\times$ PCR reaction buffer (Bioline), 1.0 μ L of MgCl₂ (50 mmol/L) (Bioline), 3 μ L (each) dNTPs (2 mmol/L) (Fisher Biotech) (2 μ L for CTX-M914), 0.5 μ L of each primer (10 mmol/L) (Invitrogen), 0.2 μ L of Taq DNA polymerase (Bioline), 20 ng of DNA

template, and sterile MilliQ water to bring the final volume to 30 μ L. PCR reactions were carried out after initial denaturation at 94 °C for 4 min; the reaction mixture was run through 30 cycles of denaturation at 94 °C for 45 s (1 min for CTXM914), annealing at 59 °C for 1 min (1 min at 55 °C for CTXM825, and 45 s at 62 °C for CTXM914), and extension at 72 °C for 1 min. Cycling was followed by a final extension incubation of 5 min at 72 °C. The PCR reaction for genes encoding OXA-10, -11, -14, -16, and -17 was performed according to Vahaboglu et al. (1998). All PCR reactions included a negative control (samples lacking a template) and a positive control (DNA extract of *E. coli* strains K6 and EC10) (Gündoğdu et al. 2012). After amplification, PCR products were separated electrophoretically and viewed as described above. Sequence analysis of the PCR amplicons was performed to confirm the tested ESBLs genes.

Detection of integron-associated *intI* genes

All representative *E. coli* strains were tested for the presence of different classes of integron-associated integrases using a multiplex PCR as described before (Dillon et al. 2005). The PCR protocol for the *intI* genes was modified to the following conditions: after initial denaturation at 94 °C for 10 min, the reaction mixture was run through 25 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 30 s, and extension at 72 °C for 1 min. Cycling was followed by a final extension incubation of 10 min at 72 °C. Negative and positive controls were included in each reaction. Positive controls were *E. coli* cells containing vectors with inserts possessing integrons of a single class only. All of the positive controls came from a subclone of those described in Long et al. (2009). These included *E. coli* uB1637 containing plasmid R388 carrying *Int3* (a class 1 integron) and *E. coli* DH5 α containing pMAQ612 carrying *Tn7* (a class 2 integron) or pSMB731 (a class 3 integron). After

Table 2. Extended-spectrum β -lactamase producing *Escherichia coli* strains detected in 2 or more samples collected from hospital wastewater over 14 weeks (W) regarded as common type (CT) and single type (ST).

Common or single type ^a	No. of strains in each type													
	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14
CT1 (10)	1	4	nd	nd	nd	nd	3	nd	nd	nd	nd	2	nd	nd
CT2 (88)	3	5	3	2	7	7	1	10	8	20	7	3	11	1
CT3 (6)	nd	nd	nd	1	2	nd	nd	3	nd	nd	nd	nd	nd	nd
CT4 (12)	1	nd	nd	3	nd	nd	nd	nd	3	2	2	nd	1	nd
CT5 (22)	3	nd	7	8	2	nd	2	nd	nd	nd	nd	nd	nd	nd
CT6 (20)	3	nd	nd	nd	nd	3	nd	5	3	nd	nd	nd	2	4
CT7 (13)	1	3	nd	nd	1	nd	nd	nd	nd	2	nd	4	2	nd
ST1 to ST27 ^b (27)	4	2	2	2	1	3	2	1	2	0	1	1	2	4
Total (198)	16	14	12	16	13	13	8	19	16	24	10	10	18	9

Note: nd, not detected.

^aNo. of isolates is in parentheses.

^bStrains belonging to single types were found only in 1 sampling occasion out of the 14 weeks sampling.

amplification, PCR products were separated electrophoretically and viewed as described above. Primer sets used for detection of all 3 classes of integrases are shown in Table 1.

Statistical analysis

Differences in the prevalence of ESBL-producing *E. coli* and the strains carrying antibiotic resistance genes in different samples were assessed using Fisher's exact test. A *p* value of <0.05 was considered statistically significant.

Results

Common types of ESBL-producing strains

In all, 252 confirmed ESBL-producing *E. coli* were isolated from hospital wastewater (*n* = 198) and from incoming influent samples of STP 1 (*n* = 29) and STP 2 (*n* = 25). Based on the combined results of the 3 typing methods, the hospital isolates were grouped into 7 common types (i.e., types with more than 2 identical isolates) and 27 single types. The lowest number of identical isolates in a common type was 6, and the highest number of identical isolates belonging to a common type was 88 (Table 2). Strains belonging to hospital common types were detected on several samples, with 1 major type (i.e., CT2) found in all 14 samples collected from hospital wastewater (Table 2). Twenty-seven (14%) of ESBL-producing isolates were found in only 1 of the 14 weekly samples and were regarded as single types (i.e., ST1–ST27) (Table 2). Typing of the isolates from STPs showed the presence of 4 common types and 3 single types in STP 1 and 3 common types and 5 single types in STP 2 (Table 3). Again, some strains were found on more than 1 occasion in samples collected from both STPs, with strains belonging to common type 1 (i.e., STP 1 CT1) found on all 4 occasions (Table 3). The mean number of ESBL-producing *E. coli* found on each sampling occasion from hospital wastewater (14.1 ± 1.2) was significantly higher than those of STPs isolates (7.3 ± 2.3 for STP 1 and 6.3 ± 2.7 for STP 2) (*p* < 0.0001).

Antibiotic resistance among untreated hospital wastewater and STP isolates

A representative of each common type isolated in each week (44 isolates from hospital wastewater samples and 17 isolates from both STPs samples) was tested for their resistance against 17 antimicrobial drugs. Among the hospital wastewater isolates, apart from cephalosporins and monobactam antibiotics, the highest resistance was observed against sulphamethoxazole and gentamicin (100% each), tetracycline (98%), and imipenem (84%) (Table 4). Generally, strains belonging to each hospital common type showed the same pattern of resistance over 14 weeks. However, there were occasional changes in the resistance of the strains in some sam-

Table 3. Extended-spectrum β -lactamase producing *Escherichia coli* strains detected in 2 or more samples collected from sewage treatment plants 1 and 2 over 4 weeks (W) regarded as common type (CT) and single type (ST).

Common or single type ^a	No. of strains in each type			
	W1	W2	W3	W4
Sewage treatment plant 1				
CT1 (11)	3	3	3	2
CT2 (6)	5	1	nd	nd
CT3 (4)	2	nd	2	nd
CT4 (5)	nd	2	nd	3
ST1 to ST3 ^b (3)	1	nd	2	nd
Sewage treatment plant 2				
CT1 (4)	nd	3	1	nd
CT2 (8)	7	nd	nd	1
CT3 (8)	1	4	3	nd
ST1 to ST5 ^b (5)	nd	2	2	1
Total (54)	19	15	13	7

Note: nd, not detected.

^aNo. of isolates is in parentheses.

^bStrains belonging to single types were found only in 1 sampling occasion out of the 4 weeks sampling with the numbers of types found in each week.

ples with the highest changes observed against amikacin, chloramphenicol, and nitrofurantoin (Table 4).

Among the STPs isolates, apart from the cephalosporins and monobactam antibiotics, the highest resistance was observed against sulphamethoxazole (100%), tetracycline (77%), nalidixic acid (47%), and norfloxacin (41%) (Table 5).

Strains isolated from hospital wastewater were significantly (*p* < 0.001) more resistant to amoxicillin – clavulanic acid, cefotetan, imipenem, gentamicin, amikacin, and tetracycline than were the STPs strains (Table 6). In contrast, strains isolated from these sites were significantly (*p* < 0.001) more resistant to ciprofloxacin, norfloxacin, and nalidixic acid than were hospital wastewater strains (Fig. 1).

Phylogenetic grouping, ESBL genes, and integrons

All weekly representative strains belonging to the 7 hospital common types as well as the 27 strains belonging to single types from hospital wastewaters (*n* = 44) (Table 2) were tested for their ESBL genes and integron classes 1 to 3 and analyzed against their phylogenetic grouping. Among the ESBL-producing strains from STPs, a similar selection was made and the 25 selected strains

Table 4. Phylogenetic groups and antibiotic resistance pattern of extended-spectrum β -lactamase producing *Escherichia coli* strains belonging to 7 common types (CT1–CT7), found in untreated hospital wastewater over 14 weeks (W1–W14) of sampling.

		Antibiotic resistance pattern																	
Common type-week ^a	Phylogenetic group	AMC	CAZ	CTX	ATM	FEP	CTT	FOX	IPM	GEN	AMK	TET	RL	CIP	NOR	NAL	NIT	CHL	
CT1-W1 (1)	B2	S	R	R	R	R	S	S	S	R	R	R	R	S	S	R*	R*	S	
CT1-W2 (4)	B2	S	S*	R	R	S*	S	S	S	R	R	R	R	S	S	R*	R*	S	
CT1-W7 (3)	B2	S	R	R	R	R	S	S	S	R	S*	R	R	S	S	S	S	S	
CT1-W12 (2)	B2	S	R	R	R	R	S	S	S	R	S*	R	R	S	S	S	S	S	
CT2-W1 (3)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R*	S	
CT2-W2 (5)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R*	S	
CT2-W3 (3)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT2-W4 (2)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT2-W5 (7)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R*	
CT2-W6 (7)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R*	
CT2-W7 (1)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R*	
CT2-W8 (10)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT2-W9 (8)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT2-W10 (20)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	R*	
CT2-W11 (7)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT2-W12 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT2-W13 (11)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT2-W14 (1)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT3-W4 (2)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R*	
CT3-W5 (2)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT3-W8 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT4-W1 (1)	A	S*	R	R	R	R	S*	S*	S*	R	S*	S*	R	S	S	S	S	S	
CT4-W4 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	R*	
CT4-W9 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	R*	
CT4-W10 (2)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT4-W11 (2)	A	S*	R	R	R	R	R	R	S*	R	S*	R	R	S	S	S	S	R*	
CT4-W13 (1)	A	R	R	R	R	R	R	R	S*	R	S*	R	R	S	S	S	S	R*	
CT5-W1 (3)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT5-W3 (7)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT5-W4 (8)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT5-W5 (2)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	R*	
CT5-W7 (2)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT6-W1 (3)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT6-W6 (3)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT6-W8 (5)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	
CT6-W9 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT6-W13 (2)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT6-W14 (4)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT7-W1 (1)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R*	S	
CT7-W2 (3)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT7-W5 (1)	A	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R*	R*	
CT7-W10 (2)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT7-W12 (4)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	
CT7-W13 (2)	A	R	R	R	R	R	R	R	R	R	S*	R	R	S	S	S	S	S	

Note: The asterisks (*) indicate differences in antibiotic resistance pattern of the strains belonging to the same common type isolated on different sampling occasions. S, sensitive; R, resistant. AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime; CTT, cefotetan; FOX, cefoxitin; IPM, imipenem; GEN, gentamicin; AMK, amikacin; TET, tetracycline; RL, sulphamethoxazole; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; NIT, nitrofurantoin; CHL, chloramphenicol.

^aNo. of isolates is in parentheses.

(17 belonging to 7 common types and 8 single types, see Table 3) were also subjected to the same tests. Distribution of phylogenetic groups, ESBL genes, and integron class 1 among representative *E. coli* strains is given in Table 7. Three of the STPs isolates showing ESBL characteristics on Mueller-Hinton agar plate did not show any positive result for the tested ESBL genes. Class 2 and 3 integrons were not found among the isolates from both sources.

Discussion

The presence of antibiotic-resistant bacteria in community and clinical settings, as well as their ecotoxic effects, continues to be a vexing problem (Kummerer 2004). Over the past decade, the prevalence of ESBL-producing *E. coli* has attracted the attention of health authorities, as reports indicate their presence not only in

patients (Bradford 2001; Rodriguez-Bano et al. 2006) but also in healthy individuals (Valverde et al. 2004) animals (Mesa et al. 2006) and the environment (Guenther et al. 2011; Ma et al. 2012). In the present study, we postulated that hospital wastewaters and STPs can serve as sources of ESBL-producing *E. coli* and that characterisation of these strains will provide a better understanding of the spread of antibiotic resistance genes and their sources outside hospitals and healthcare facilities. To increase the chance of detecting minor clones of ESBL-producing *E. coli* that may be found in hospitals, we decided to collect our samples from the hospital wastewater and over a long period (14 weeks). Similarly, to detect ESBL-producing *E. coli* originating from the community, we selected 2 STPs that did not receive wastewaters from any hospitals.

Table 5. Phylogenetic groups and antibiotic resistance pattern of extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* strains belonging to 4 common types (CT1–CT4) found in sewage treatment plant 1 and 3 common types (CT1–CT3) found in sewage treatment plant 2 over 4 weeks (W1–W4) of sampling.

		Antibiotic resistance pattern																
Common type-week ^a	Phylogenetic group	AMC	CAZ	CTX	ATM	FEP	CTT	FOX	IPM	GEN	AMK	TET	RL	CIP	NOR	NAL	NIT	CHL
Sewage treatment plant 1																		
CT1-W1 (5)	B1	R	R	R	R	R	R	R	S	S	S	S	R	S	S	S	S	S
CT1-W2 (1)	B1	R	R	R	R	R	R	R	S	S	S	S	R	S	S	S	S	S
CT2-W1 (3)	D	S*	R	R	R	R	S	R	S	S	S	R	R	R	R	R	S	S
CT2-W2 (3)	D	S*	R	R	R	R	S	R	S	S	S	R	R	R	R	R	S	S
CT2-W3 (3)	D	R	R	R	R	R	S	R	S	S	S	R	R	R	R	R	S	S
CT2-W4 (2)	D	R	R	R	R	R	S	R	S	S	S	R	R	R	R	R	S	S
CT3-W2 (2)	A	S	R	R	R	R	S	S	S	S	S	R	R	S	S	R	S	R
CT3-W4 (3)	A	S	R	R	R	R	S	S	S	S	S	R	R	S	S	R	S	R
CT4-W1 (2)	A	R	R	R	R	R	S	R	R	S	S	R	R	S	S	S	S	R
CT4-W3 (2)	A	R	R	R	R	R	S	R	R	R*	S	R	R	S	S	S	S	R
CT1-W2 (3)	B1	S	S	R	R	R	S	S	S	S	S	S	R	S	S	S	S	S
CT1-W3 (1)	B1	S	S	R	R	R	S	S	S	R*	S	S	R	S	S	S	S	S
CT2-W1 (7)	B2	S	S	R	R	S	S	S	S	R	S	R	R	S	S	S	S	S
CT2-W4 (1)	B2	S	S	R	R	S	S	S	S	S*	S	R	R	S	S	S	S	S
Sewage treatment plant 2																		
CT3-W1 (1)	A	S	R	R	R	R	S	S	S	R	R	R	R	S	R	R	R	R
CT3-W2 (4)	A	S	R	R	R	R	S	S	S	R	R	R	R	S	R	R	S*	S*
CT3-W3 (3)	A	S	R	R	R	R	S	S	S	S*	S*	R	R	S	R	S*	S*	S*

Note: The asterisks (*) indicate differences in antibiotic resistance pattern of the strains belonging to the same common types isolated on different sampling occasions. S, sensitive; R, resistant. AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime; CTT, cefotetan; FOX, cefoxitin; IPM, imipenem; GEN, gentamicin; AMK, amikacin; TET, tetracycline; RL, sulphamethoxazole; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; NIT, nitrofurantoin; CHL, chloramphenicol.

^aNo. of isolates is in parentheses.

Table 6. Distribution of phylogenetic groups, extended-spectrum β -lactamase (ESBL) genes, and integron class 1 among 96 representative (71 from hospital wastewaters and 25 from sewage treatment plants) ESBL-producing *Escherichia coli* strains.

Source	No. of isolates (%)									
	Phylogenetic group				β -Lactamase gene				Integron	
	A	B1	B2	D	TEM	SHV	CTX-M	OXA	<i>intI1</i>	
Hospital wastewater	58 (82)	6 (9)	3 (4)	4 (5)	57 (80)*	52 (73)*	8 (11)	nd	53 (75)	
Sewage treatment plants	10 (40)	8 (32)	2 (8)	5 (20)	4 (16)	nd	20 (80)**	nd	19 (76)	

Note: nd, not detected. *, $p < 0.001$; **, $p < 0.0001$.

Resistance to several antimicrobial agents in *E. coli* isolated from hospital wastewaters and STPs have been reported before (Diwan et al. 2010; Kummerer 2009b). However, little data are available on ESBL-producing *E. coli* in hospital wastewater and community STPs. Our results indicated the presence of different clonal groups of ESBL-producing *E. coli* strains in both the hospital wastewater and the studied STPs, some of which were resistant to many of the tested antimicrobial agents including imipenem. Hospitals have been known as an important source of resistant bacteria, and in this study, we showed a much higher percentage of ESBL-producing *E. coli* in hospital wastewaters than in the STPs. While there is uncertainty as to whether the presence of antibiotics in the aquatic environment would result in the development of resistance in bacteria (Diwan et al. 2010), it has been shown that antibiotic-resistant bacteria might be selected or favoured by the presence of a high concentration of antibiotic residues in hospital wastewater (Kummerer 2004; Baquero et al. 2008). Despite the fact that β -lactam antibiotics are one of the major antibiotic groups prescribed in hospitals, they have only been detected in a limited number of studies and to a lesser extent in hospital wastewater (Watkinson et al. 2009). This is probably due to easy degradation of the β -lactam ring, its high metabolic rate, and the process of decarboxylation (Kummerer 2009b). The presence of a high level of ESBL-producing *E. coli* in the hospital wastewater in our study could mainly be due to the persistence of these strains in hospital

environment and their regular discharge in hospital wastewater. Wiethan et al. (2001) demonstrated that there is no correlation between the hospital input and the presence of resistant bacteria in downstream STP. These researchers also claimed that hospitals are not the main source of resistant bacteria in STPs. Contrary to this, Romling et al. (1994) indicated that multidrug resistance bacteria may be selected for in hospitals and passed into wastewater. Our study suggests that these resistant strains can become dominant in hospitals, as they constituted a high percentage of multidrug-resistant *E. coli* strains in hospital wastewater. Using a combination of 3 typing methods to identify clonal groups of ESBL-producing strains in both sources, we found that the majority (86%) of the isolates in hospital wastewater belonged to a few common types. Strains of these common types were frequently found in the weekly samples, with the most common clonal type, i.e., CT2, constituting 44% (88 out of 198 isolates tested) of the hospital strains.

In this study, we also looked for the presence and prevalence of ESBL-producing *E. coli* in STPs that did not receive wastewater from hospitals, to obtain an estimation of the prevalence of ESBL-producing strains in community STPs. Due to logistical problems we limited our sampling to only 4 weeks but extended our search to 2 STPs. We noticed diverse types of ESBL-producing strains, most of which were found only on 1 sampling occasion (S-types). This was somehow expected, as community STPs normally serve a

Fig. 1. Percentage of antibiotic-resistant extended-spectrum β -lactamase (ESBL) producing strains found in hospital wastewaters (solid bars) and sewage treatment plants (open bars). Only antibiotics that showed significant differences between 2 sources are shown (a cut-off of $p < 0.05$ was used to define statistical significance). AMC, amoxicillin – clavulanic acid; CTT, cefotetan; IPM, imipenem; GEN, gentamicin; AMK, amikacin; TET, tetracycline; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid. *, $p < 0.001$.

high population ranging from hundreds of thousands to over a million residents (Payment et al. 2001), and as such, the population of *E. coli* strains in these treatment plants should be quite diverse (Escobar-Paramo et al. 2004). Despite this, we found the presence of the same clonal types (4 in STP 1 and 3 in STP 2) on more than 1 occasion, indicating that either these strains are commonly introduced into the STP or they have a better ability to survive the STP or a combination of both. This finding also indicates that the community can also serve as a common source of ESBL-producing *E. coli*, with some clonal types being more common or persistent in the community and (or) STPs.

ESBL-producing strains are known to be increasingly associated with resistance to non- β lactam antimicrobials due to mechanisms such as expression of the related genes on plasmid (Poirel et al. 2006) or loss of an outer membrane porin protein (Elliott et al. 2006). Strains isolated in our study were also highly resistant to unrelated antimicrobial drugs, including imipenem (carbapenems), ceftazidime, and β -lactam – β -lactamase inhibitor combination drugs. To our knowledge, this is the highest resistance against imipenem found for ESBL-producing *E. coli* isolated from hospital wastewater and STPs. The carbapenem antibiotics such as imipenem have the broadest antibacterial spectra of all available β -lactams (Zhanel et al. 2007). They are also more stable than any available cephalosporin to the AmpC-type β -lactamases and ESBLs (Edwards and Betts 2000). However, according to our study this excellent drug might not be an attractive choice anymore because of the high (92%) level of imipenem resistance among *E. coli* strains found in hospital wastewaters. These strains were also ESBL producers and showed a high frequency of resistance to β -lactam antibiotics and combinations of β -lactams with commercially available β -lactamase inhibitors. However, it has been suggested that seasonal changes have a perceptible effect on both antibiotic use and resistance activity of bacteria in the environment (Mesa et al. 2006). Considering the long duration of our sampling from hospital wastewater (14 weeks) it is possible that seasonal change has in part affected residue of antimicrobial agents as well as bacterial populations in our samples.

In general, strains found in hospital wastewaters were significantly more resistant to β -lactamase inhibitor combination drugs cephamycin and carbapenem than were STP strains. This might be the result of a high usage of these antibiotics in hospitals. In fact, these antimicrobial agents are among the most commonly

used drugs in human medicine. According to the report of the Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR 1999), between 1992 and 1997 the import of active ingredient for β -lactam inhibitors, carbapenems, and cephalosporins into Australia averaged 40 000 kg per year. The figure for veterinary medicine in this country was around 400 kg per year.

Epidemiological studies have revealed a strong relationship between fluoroquinolone resistance and ESBL production in *Enterobacteriaceae* (Hirakata et al. 2005). However, in our study this relationship was found only among STP strains. Fluoroquinolones have never been licensed to be used in animal food production in Australia. Therefore, the presence of higher fluoroquinolone resistance among STP strains must presumably be the result of use of this drug in human medicine.

It is well known that CTX-M is the most common type of ESBL produced by *E. coli* strains. Interestingly, we found that the majority of strains isolated from hospital wastewaters (73%) carried SHV-type ESBL gene, whereas none of the STP strains carried this gene. This could possibly be due to the high rate of exchange of resistance genes via mobile genetic elements (Schluter et al. 2003) in hospital bacterial populations. It has to be noted that this type of gene transfer is also important in hospital effluents, because in such environments the possibility of gene exchange is increased as a result of higher density and greater contact between these microorganisms, which allows for an increased rate of horizontal transfer of these genes. Therefore, the phenotypes found in the samples collected from hospital wastewaters may be representative of the phenotypes circulating in the hospital environment.

In conclusion, we demonstrated the presence of certain clonal groups of ESBL-producing *E. coli* in both hospital wastewaters and STPs. These strains were also resistant to several non- β -lactams and (or) an unrelated subgroup of β -lactam antibiotics. Among hospital strains, the majority belonged to phylogenetic group A, but the STP strains belonged to the 2 major groups A and B1. Furthermore, despite the commonly reported CTX-M-type ESBL-producing strains, we found that majority of the *E. coli* strains isolated from untreated hospital wastewater carried SHV-type ESBL.

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