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Emergence of extended-spectrum β-lactamase and fluoroquinolone resistance genes among Irish multidrug-resistant isolates

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Abstract

This study sought to identify mechanisms behind resistance to third-generation cephalosporins and ciprofloxacin in Irish multidrug-resistant Enterobacteriaceae isolates. The most prevalent extended-spectrum β-lactamase genes identified were bla_{SHV-12} and $bla_{CTX-M-15}$. These were associated with the fluoroquinolone resistance genes *aac(6′)-IB-cr*, *qnrA*, and *qnrB*, not previously reported in Irish isolates.

Keywords: ESBL; $bla_{CTX-M-15}$, *aac(6′)-Ib-cr*; Enterobacteriaceae; Ireland

In recent years, the emergence of multidrug-resistant (MDR) Gram-negative pathogens, particularly extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, has risen dramatically and caused a global health-care problem (Cantón and Coque, 2006; Coque et al., 2008a, 2008b).

Increased antibiotic resistance among Gram-negative isolates has been observed at the Mercy University Hospital (MUH), Cork, Ireland, a 350-bed teaching hospital in the south of Ireland. Between 2004 and 2007, resistance to third-generation cephalosporins, ceftazidime, and cefotaxime rose from 2% to 6% and 1% to 7%, respectively, and ciprofloxacin resistance rose from 5% to 15%. This increase is similar to previously published data showing rising non-susceptibility to cephalosporins and ciprofloxacin among Enterobacteriaceae throughout Europe (European Antimicrobial Resistance Surveillance System, 2007; Livermore et al., 2008; Murchan et al., 2006). However, data regarding the dissemination of resistance mechanisms among MDR Enterobacteriaceae in the Republic of Ireland are limited. To investigate which mechanisms caused the increased resistance to ciprofloxacin and third-generation cephalosporins, we undertook retrospective characterization of MDR Enterobacteriaceae bacteria isolated at the MUH.

Over three 6-month surveillance intervals (period A, 01.01.2004–30.06.2004; period B, 01.01.2006–30.06.2006; period C, 01.10.2006–31.03.2007), 3910 Gram-negative bacteria were isolated from patients at the MUH, which were identified using API 20E test strips (Bio-Merieux Hampshire, UK). All bacterial strains were tested for sensitivity to ampicillin, amoxicillin–clavulanic acid, cephalothin, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, amikacin, meropenem, piperacillin–tazobactam, cefepime, and aztreonam by disk diffusion in accordance with Clinical and Laboratory Standards Institute (2007) guidelines. Isolates exhibiting nonsusceptibility to ≥5 of these antibiotics were considered MDR. Seventy-two isolates (1.8%) met these selection criteria. Six MDR nonfermentative isolates were identified (4 *Stenotrophomonas maltophilia*, 1 *Pseudomonas aeruginosa*, 1 *Burkholderia cepacia*) but were omitted from further analysis, because this study focused on MDR Enterobacteriaceae. Among them, *Escherichia coli* was the most frequently identified species (38%, 25/66) followed by *Enterobacter cloacae* (32%, 21/66) and *Klebsiella pneumoniae* (11%, 7/66). All exhibited cephalosporin nonsusceptibility of which 53% (35/66) exhibited an ESBL phenotype (E-test; AB Biodisk, Sweden) (Table 1). The overall prevalence of ciprofloxacin resistance was 77% (51/66)

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Table 1
Distribution of antibiotic resistance among MDR Enterobacteriaceae strains used in this study

Species	No. of isolates	ESBL positive[a]	No. of nonsusceptible isolates								
			AMC	CTX	CAZ	CIP	TZP	CPM	GM	AK	MEM
E. coli	25	20 (80)	25 (100)	25 (100)	23 (92)	23 (92)	10 (40)	14 (56)	9 (36)	1 (4)	0
E. cloacae	21	7 (33)	21 (100)	21 (100)	21 (100)	12 (57)	16 (76)	3 (14)	7 (33)	0	0
Enterobacter spp.	3	0	3 (100)	3 (100)	3 (100)	1 (33)	2 (66)	1 (33)	1 (33)	0	0
K. pneumoniae	7	7 (100)	1 (14)	7 (100)	7 (100)	7 (100)	1 (14)	0	7 (100)	0	0
Klebsiella oxytoca	3	1 (33)	2 (66)	0	2 (66)	1 (33)	2 (66)	0	2 (66)	0	0
Serratia marcescens	4	0	4 (100)	4 (100)	2 (50)	4 (100)	4 (100)	0	0	0	0
Morganella morganii	2	ND	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	ND	0	0	0
Raoultella ornithinolytica	1	ND	1 (100)	0	0	1 (100)	1 (100)	ND	0	0	0
Total	66	35 (53)	59 (89)	62 (94)	60 (91)	51 (77)	37 (56)	18 (27)	26 (39)	1 (2)	0

AMC = amoxicillin + clavulanic acid; CTX = cefotaxime; CAZ = ceftazidime; CIP = ciprofloxacin; TZP = piperacillin + tazobactam; GM = gentamicin; CPM = cefepime; AK = amikacin; MEM = meropenem; ND = not determined. Percentages are given in brackets. All MDR isolates were also resistant to both ampicillin and cephalothin.

[a] Identified phenotypically using Etest ESBL strips (AB Biodisk).

among MDR Enterobacteriaceae and 83% among MDR Enterobacteriaceae, which were also ESBL positive (29/35).

Strain diversity among MDR *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. was assessed by randomly amplified polymorphic DNA (RAPD) analysis using the previously described primer-1254 (Mugnaioli et al., 2006). The diversity of RAPD profiles generated showed heterogeneity among *Enterobacter* spp. and *Klebsiella* spp. isolates, thereby excluding clonal spread of 1 particular MDR strain. Several smaller (<3 isolates) but also 1 larger related group (12 isolates) was identified among *E. coli* isolates. However, further typing using BOX, ERIC, and REP-specific primers (Rademaker, 2004) identified differences between the 12 strains, excluding the presence of a recurrent hospital-acquired clone. Moreover, 6 of these strains were isolated from patients within 48 h of admission, suggesting that community acquisition and interstrain antibiotic resistance profiles differed. Therefore, overrepresentation of resistance genes due to the presence of clonal hospital-acquired strains was minimal.

All MDR *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. isolates were screened for the presence of TEM-, SHV-, CTX-M-, AmpC-, and OXA-family β-lactamase genes using previously described primer sets (Arlet et al., 1995, 1997; Pérez-Pérez and Hanson, 2002; Steward et al., 2001; Woodford et al., 2006). Sequence analysis was performed on bla_{TEM}, bla_{SHV}, bla_{AmpC}, and bla_{CTX-M} polymerase chain reaction (PCR) products to identify specific β-lactamase genes. TEM-family β-lactamase genes were highly represented among MDR Enterobacteriaceae. Interestingly, sequence analysis revealed the absence of TEM-family ESBLs, because only non-ESBL variants including bla_{TEM-1}, bla_{TEM-2}, bla_{TEM-30}, and bla_{TEM-39} were identified (Table 1). However, TEM-family genes were associated with $bla_{CTX-M-15}$ and bla_{SHV-12}, which were the dominant ESBLs detected (Table 2). For 2 out of the 35 ESBL-positive isolates, we were unable to identify the gene responsible for ESBL production. No TEM- or SHV-family ESBL variants were detected in these strains, nor were CTX-M-, AmpC-, GES-, VEB- or PER-type β-lactamase genes. The mechanism of cephalosporin resistance in these strains is the subject of further investigation.

Bla_{SHV-12} is widely disseminated among MDR Enterobacteriaceae in Europe (Coque et al., 2008a, 2008b). It was detected with highest prevalence among *Klebsiella* spp. (80%, 8/10) followed by *Enterobacter* spp. (50%, 12/24) and *E. coli* (8%, 2/25). Although half of the *Enterobacter* spp. isolates harbored bla_{SHV-12}, only 29% (7/24) were phenotypically ESBL positive (Table 2). Up-regulation of the chromosomal AmpC β-lactamase of *Enterobacter* spp. isolates results in resistance to third-generation cephalosporins and can also confound detection of ESBLs (Szabó et al., 2005). Although a combination of cefepime, which should be relatively unaffected by high-level AmpC expression, and clavulanic acid was used to detect ESBL production, false-negative results were obtained. Thus, MDR *Enterobacter* spp. represent a significant problem at this institution, confounding selection of pertinent treatment options based on susceptibility data.

$Bla_{CTX-M-15}$ was detected in 64% (16/25) of MDR *E. coli*. The prevalence of $bla_{CTX-M-15}$-producing *E. coli* increased over the 3 collection intervals (period A, 1 strain; period B, 3 strains; period C, 11 strains). Sixty-three percent (10/16) of the $bla_{CTX-M-15}$-positive *E. coli* identified were isolated within 48 h of hospital admission, suggesting community acquisition, although 3 patients were nursing home residents that have been identified as reservoirs for ESBL-producing *E. coli* (Rooney et al., 2009). The increased prevalence of $bla_{CTX-M-15}$-positive *E. coli* coupled with the evidence for community acquisition suggests an endemic situation in this region.

Fluoroquinolones are used to combat infections caused by ESBL-producing Enterobacteriaceae. However, increased fluoroquinolone resistance has now been associated with ESBL isolates (Falagas and Karageorgopoulos, 2009). This increase has apparently occurred in parallel with the emergence of plasmid-mediated fluoroquinolone resistance mechanisms (Coque et al., 2008a, 2008b; European

Table 2
Distribution of β-lactam and fluoroquinolone resistance genes among MDR Enterobacteriaceae isolated at MUH

	Species				Total	% of MDR Enterobacteriaceae
	E. coli	*Enterobacter* spp.	*Klebsiella* spp.	Other		
Total isolates	25	24	10	7	66	
Nonclonal isolates[a]	17	18	9	ND[b]		
ESBL-positive isolates	20	7	8	ND	35	53
β-lactam resistance genes[c]						
bla_{TEM-1}	3 (1)	2 (0)	1 (0)	ND	6 (1)	9.1
bla_{TEM-2}	0	1 (0)	0	ND	1 (0)	1.5
bla_{TEM-30}	1 (0)	1 (0)	0	ND	2 (0)	3.0
bla_{TEM-39}	1 (0)	0	0	ND	1 (0)	1.5
bla_{TEM-1} bla_{OXA-1}	0	0	1 (0)	ND	1 (0)	1.5
bla_{TEM-1} bla_{SHV-11}	1 (1)	0	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{SHV-12}}$	1 (1)	3 (1)	2 (2)	ND	6 (4)	9.1
bla_{TEM-1} $\underline{bla_{SHV-12}}$ bla_{CMY-2}	1 (1)	0	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{SHV-12}}$ bla_{OXA-1}	0	0	3 (3)	ND	3 (3)	4.6
$\underline{bla_{SHV-12}}$	0	4 (2)	0	ND	4 (2)	6.1
$\underline{bla_{SHV-12}}$ $\underline{bla_{CTX-M-25}}$	0	1 (1)	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{CTX-M-15}}$	4 (4)	0	0	ND	4 (4)	6.1
bla_{TEM-1} $\underline{bla_{CTX-M-15}}$ bla_{OXA-1}	1 (1)	0	0	ND	1 (1)	1.5
bla_{TEM-1} bla_{CMY-2}	1 (0)	0	0	ND	1 (0)	1.5
None	0	8 (0)	0	ND	8 (0)	12.1
β-lactam + FQ resistance genes[c]						
bla_{TEM-1} $\underline{bla_{CTX-M-15}}$ bla_{OXA-1} *aac(6′)-Ib-cr*	9 (9)	0	0	ND	9 (9)	13.6
bla_{TEM-1} $\underline{bla_{CTX-M-15}}$ *aac(6′)-Ib-cr*	1 (1)	0	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{CTX-M-15}}$ bla_{OXA-1} *aac(6′)-Ib-cr qnrA*	1 (1)	0	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{SHV-12}}$ *qnrA*	0	3 (2)	0	ND	3 (2)	4.6
$\underline{bla_{SHV-12}}$ *qnrA*	0	1 (1)	0	ND	1 (1)	1.5
bla_{TEM-1} $\underline{bla_{SHV-12}}$ bla_{OXA-1} *qnrB*	0	0	2 (2)	ND	2 (2)	3.0
bla_{TEM-1} $\underline{bla_{SHV-12}}$ *qnrB*	0	0	1 (1)	ND	1 (1)	1.5

[a] Number of unique genomic DNA fingerprints observed among each species.
[b] ND = not determined.
[c] The number of phenotypically ESBL-positive isolates is indicated in brackets. Identified ESBL genes are underlined to distinguish them from non-ESBL genes.

Antimicrobial Resistance Surveillance System, 2007; Nordmann and Poirel, 2005). Therefore, all 72 isolates were screened for the *qnrA*, *qnrB*, *qnrS*, and *qepA* fluoroquinolone resistance genes and the *aac(6′)-Ib* family acetyltransferase gene using previously described primer sets (Cattoir et al., 2007; Périchon et al., 2007; Robicsek et al., 2007). The fluoroquinolone-modifying *aac(6′)-Ib-cr* variant was subsequently identified by amplicon sequencing.

The *qnr* genes *qnrA* and *qnrB* were detected in association with bla_{SHV-12} in *Enterobacter* (4/24, 17%) and *Klebsiella* spp. (3/11, 27%) isolates, respectively. The *aac(6′)-Ib-cr* gene was detected exclusively in *E. coli* (11/25, 44%). Among detected transferable fluoroquinolone resistance genes, an increase in *aac(6′)-Ib-cr* prevalence was observed over the 4 collection intervals in contrast to detected *qnr* genes: period A, 7 genes (1 *aac(6′)-Ib-cr*, 6 *qnr*); period B, 4 genes (3 *aac(6′)-Ib-cr*, 1 *qnr*); period C, 8 genes (7 *aac(6′)-Ib-cr*, 1 *qnr*). Six of the identified *aac(6′)-Ib-cr*–positive isolates were isolated within 48 h of hospital admission suggesting community acquisition. Several recent studies have indicated that the *aac(6′)-Ib-cr* gene is often associated with $bla_{CTX-M-15}$ (Jones et al., 2008; Nordmann and Poirel, 2005). Indeed, all *aac(6′)-Ib-cr*–positive isolates also harbored the $bla_{CTX-M-15}$ gene, suggesting that both are present on the same plasmid (Table 2).

Conjugation experiments were performed to identify if β-lactam and ciprofloxacin resistance were cotransferred to a susceptible *E. coli* J53 rifampicin-resistant recipient. Transconjugants were selected on 300 μg/mL rifampicin and 3 μg/mL cefotaxime. The transfer of cephalosporin resistance was observed for 13/20 ESBL-positive *E. coli* isolates including 9/11 *aac(6′)-Ib-cr* and $bla_{CTX-M-15}$ coproducing strains. Among these isolates, cefotaxime and low-level ciprofloxacin resistance were always cotransferred with resistance to tobramycin and kanamycin, consistent with the cotransfer of $bla_{CTX-M-15}$ and *aac(6′)-Ib-cr*. Both genes were detected in transconjugants by PCR, further suggesting that both genes are present on a common transmissible plasmid. This was substantiated by the detection of a single plasmid in 7 of the 9 $bla_{CTX-M-15}$ and *aac(6′)-Ib-cr* coharboring transconjugants. In 2 of the 9 transconjugants, 2 plasmids were present, confounding

interpretation of the linkage between $bla_{CTX\text{-}M\text{-}15}$ and *aac (6′)-Ib-cr* on a common plasmid in these strains.

The dissemination of both $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$ among clonally diverse Enterobacteriaceae has contributed to increased cephalosporin resistance at this institution. The detection of *aac(6′)-Ib-cr* in $bla_{CTX\text{-}M\text{-}15}$-positive *E. coli* and the detection of *qnrA* and *qnrB* among MDR Enterobacteriaceae represent the first report of transferable fluoroquinolone resistance mechanisms among Irish clinical isolates.

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