Prevalence of transferable $bla_{CTX-M-15}$ from hospital- and community-acquired Klebsiella pneumoniae isolates in Scotland

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Objectives: This study was performed to investigate the prevalence and genetic characteristics of transferable $bla_{CTX-M-15}$ from hospital- and community-acquired Klebsiella pneumoniae isolates in Scotland.

Methods: A total of 219 clinical isolates of $K. pneumoniae$ collected in 2006 and 2007 at the Royal Infirmary of Edinburgh, Scotland, were tested for antimicrobial susceptibility by the agar double dilution method. PCR and sequencing were used to detect $bla_{CTX-M}$, $bla_{TEM}$, $bla_{SHV}$ and $qnr$ genes. Clonality of the isolates was assessed by PFGE.

Results: Sixteen (7.3%) isolates were found to be producers of CTX-M-15 extended-spectrum $\beta$-lactamases (ESBLs), of which two isolates (12.5%) were reported to be from patients with community-acquired infections. The IS$Ecp1$ was detected by sequencing 48 nucleotides upstream of $bla_{CTX-M-15}$ in all isolates but one. A total of one to two plasmids, ranging in size from $\approx 40$ to 210 kb, were observed per strain. By a PCR-based replicon typing method, plasmids carrying $bla_{CTX-M-15}$ were assigned to IncFII or IncN types. Sequencing and PCR analysis revealed the presence of complex class 1 integrons in all isolates but one. Two isolates positive for class 1 integrons were positive for class 2 integrons as well. Five different clones of CTX-M-15-producing isolates were identified by PFGE.

Conclusions: This work reports the emergence of hospital- and community-acquired CTX-M-type enzymes in the Edinburgh area of Scotland.

Keywords: extended-spectrum $\beta$-lactamases, cephalosporin resistance, IncN plasmid, PFGE, integrons

Introduction

Klebsiella spp. are opportunistic human pathogens that can be isolated from various animal and human clinical specimens. Klebsiella pneumoniae is a Gram-negative bacterium, which causes both hospital- and community-acquired infections, such as pneumonia, urinary tract infections, septicaemia, soft tissue infections, liver abscesses and bacterial meningitis. 1,2 Although extended-spectrum $\beta$-lactamases (ESBLs) have been detected in a wide variety of Gram-negative bacteria, K. pneumoniae is still an important ESBL producer, not only in the nosocomial setting but also in the community. 3

The CTX-M $\beta$-lactamases constitute one of the most rapidly growing ESBL families. Outbreaks of the CTX-M enzyme have been described in bacteria from Africa, Europe, South America and Asia. 4–6 The CTX-M family is subclassified into five groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25), in which the members of each group share 94% identity. 7 CTX-M types have evolved through the escape of chromosomal genes from Kluyvera ascorbata and Kluyvera georgiana. 8–11 The ability of insertion sequences IS$Ecp1$ and IS$CR1$, phage-related elements, and plasmids to mobilize and to promote the expression of $\beta$-lactamase genes may explain the current spread of CTX-M-type enzymes worldwide. 7,12–14

CTX-M-15 was first detected in isolates from India. 15 It is derived from CTX-M-3 by an Asp240Gly substitution, which increased catalytic activity against ceftazidime. 16 Since 2003, Escherichia coli isolates with the CTX-M-15 ESBL have become widely distributed throughout the UK. Five major related E. coli strains (A–E) have been identified by PFGE, and they all belong to sequence type (ST) ST131 and serotype O25:H4. Strain A is the most distributed lineage in the UK. 17,18 Outbreaks of CTX-M-15-producing K. pneumoniae isolates have been described recently in some European countries, such as those reported in Scandinavia and Slovenia. 19,20 In Spain, only a single epidemic clone characterized by PFGE was found from a single institution.
in Madrid\textsuperscript{1} whereas in another study, Oteo et al.\textsuperscript{21} identified seven PFGE clusters corresponding to seven different \textit{K. pneumoniae} STs collected from five Spanish hospitals.

Little is known about the population structure of CTX-M-15-producing \textit{K. pneumoniae} causing outbreaks in the UK, in comparison with CTX-M-15-producing \textit{E. coli}.

**Materials and methods**

**Bacterial strains**

A total of 219 clinical isolates of \textit{K. pneumoniae} consecutively collected in 2006 and 2007 at the Royal Infirmary of Edinburgh, Scotland, were processed. Identification of the phylogenetic types of strains was confirmed by gyr\textit{A} PCR–restriction fragment length polymorphism (RFLP) using restriction enzymes HincII, TaqI and HaeIII, as previously described.\textsuperscript{22}

**Antimicrobial susceptibility testing**

Isolates were tested for antimicrobial susceptibility by the agar double dilution method (MIC) using Iso-Sensitest agar (Oxoid). The antibiotics amoxicillin, piperacillin, nalidixic acid, ciprofloxacin, cefuroxime, cefalexin, ceftriaxone, cefotaxime, ceftazidime, aztreonam, cefoxitin and meropevtac were tested according to manufacturer’s instructions. The resulting PCR products were subsequently tested for ESBL production by double disc and combination disc diffusion methods.

**PCR amplification and sequencing**

Genes coding for a CTX-M phenotype were screened by PCR, initially with the universal primers CTX-M\_F and CTX-M\_R\textsuperscript{24} and then with primers specific for the \textit{bla}\textsubscript{CTX-M-1} group.\textsuperscript{25} The genes coding for \textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV}, \textit{qnrA}, \textit{qnrB} and \textit{qnrS} were amplified by PCR from genomic DNA using oligonucleotide primers, as described before.\textsuperscript{26,27} PCR products were purified with a Qiagen purification kit according to the manufacturer’s instructions. The resulting PCR products were sequenced by the Sanger method using an ABI 373A DNA sequencer (PE Applied Biosystems). The BLAST and FASTA programs of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) were used to search databases for similar nucleotide and amino acid sequences.

**Isoelectric focusing**

The presence of active CTX-M \(\beta\)-lactamase was confirmed by the identification of a characteristic band (pl 8.6) in comparison with the pl values of known \(\beta\)-lactamases [TEM-2 (pl 5.6), SHV-1 (pl 7.6) and SHV-3 (pl 7)] (data not shown).

**PFGE**

The clonal relationship between isolates was studied by PFGE using 30 U of XbaI (Promega, UK), as previously described.\textsuperscript{28} Genotypic relatedness was evaluated with BioNumerics software using the Dice coefficient and arithmetic mean (UPGMA) algorithms.

**Plasmid characterization**

The plasmids were extracted with a midi-kit (Qiagen), according to manufacturer’s instructions. Plasmid DNA electrophoresis was performed in 0.7% agarose. For fingerprinting analysis, plasmid DNA was digested with the EcoRI and PstI restriction enzymes (Promega, UK). Plasmid size and content were determined using S1 nuclease-digested genomic DNA. Plasmids were classified according to their incompatibility group with the PCR-based replicon typing described by Carattoli et al.\textsuperscript{29} The primer pairs targeting FIA, FIB, FIC, I2, I1, I/M, N, A/C and FII replicons were used in separate PCRs.

**\(\beta\)-Lactam resistance transfer assays**

Mating experiments were performed with \textit{E. coli} J62-2 (Rif\textsuperscript{R}). Cultures of each donor and the recipient strain were grown in Luria-Bertani (LB) broth (Sigma), and then mixed in the ratio of 1:4 and incubated for 5 h at 37\(^{\circ}\)C. Transconjugants were then selected on MacConkey agar plates containing rifampicin (150 mg/L) and cefotaxime (2 mg/L). For transformation, plasmid DNA was transferred by electroporation into \textit{E. coli} DH5\textit{a} cells. Transformants were plated on LB agar supplemented with cefotaxime (2.5 mg/L).

**Genetic environment of \textit{bla}\textsubscript{CTX-M-15} genes**

The genetic organization of the \textit{bla}\textsubscript{CTX-M} genes was investigated by PCR and sequencing of the regions surrounding these genes. Detection of the upstream IS\textsubscript{Ecp1} insertion sequence was investigated using CTX-M\_R reverse and Prom\_ primers.\textsuperscript{22} The internal IS\textsubscript{262} was determined using primers, as described before.\textsuperscript{18} \textit{K. pneumoniae} isolates were screened for the presence of int\textsubscript{I1}, sul\textsubscript{1} and qac\textsubscript{E}\textsubscript{A\textsubscript{1}}, which represent class 1 integrons, and for int\textsubscript{I2} integrase encoded on class 2 integrons.\textsuperscript{30} To characterize the presence of inserted gene cassettes within the variable region, the primer pair S\_CS and 3\_CS for class 1 integrons was used.\textsuperscript{31}

**Results and discussion**

**Characterization of \(\beta\)-lactamases**

The overall frequency of ESBL producers observed in this study was 32/219 (14.61%). By PCR, CTX-M genes were detected in 16 (7.3%) isolates of \textit{K. pneumoniae}. All isolates were identified as CTX-M-15 by the results of sequence analysis of the deduced amino acid sequences.

In this study, we identified only the CTX-M-15 enzyme, which is the most common CTX-M type in the UK. The first CTX-M ESBL in the UK was described in 2000, in one isolate of \textit{Klebsiella oxytoca}.\textsuperscript{32} During June 2001, in the first hospital outbreak, 36 \textit{K. pneumoniae} isolates from 33 patients from 15 different wards and departments around Birmingham (UK) were described as CTX-M-producing isolates.\textsuperscript{33} In 2001, a survey examined >900 \textit{E. coli} from 28 hospitals in the UK and Ireland, and recorded 4 isolates with CTX-M enzymes.\textsuperscript{34} In 2004, Woodford et al.\textsuperscript{18} identified an epidemic clone of \textit{E. coli} producing CTX-M-15 that had become widely distributed throughout the UK.

As shown in Table 1, all isolates harbouring the CTX-M enzyme were found to carry SHV-11, SHV-12 or SHV-5 \(\beta\)-lactamases by PCR. Moreover, all isolates but one were found to express TEM-1. This association is frequent and has already been described.\textsuperscript{35} In this study, 10 \textit{qnrB} genes were associated with CTX-M-15. The co-presence of \textit{qnrB} and ESBL genes on the same plasmid has been regularly reported.\textsuperscript{36}

Using gyr\textit{A} PCR–RFLP to identify the phylogenetic types of strains, we assigned all isolates to \textit{K. pneumoniae} phylogenetic group 1.
### Table 1. Characterization of CTX-M-producing *K. pneumoniae* isolates

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<th>TEM</th>
<th>SHV</th>
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### β-Lactam susceptibility profile

The antimicrobial susceptibility patterns showed that the strains harboured CTX-M-15 ESBLs characterized by high resistance to most cephalosporins. Resistance of CTX-M-producing isolates to cefotaxime, ceftazidime, ceftriaxone, nalidixic acid, ciprofloxacin, cefotaxin and meropenem was found to be 100%, 100%, 100%, 93.8%, 93.8%, 81.3% and 0%, respectively. This high resistance to most antibiotics is explained by the finding that *blaCTX-M* genes are commonly found on plasmids carrying genes that confer resistance to multiple antibiotics, including aminoglycosides, chloramphenicol, sulphonamides, trimethoprim and tetracycline. CTX-M-15 ESBLs confer high-level resistance to ceftazidime (MIC range: 64 to >128 mg/L). Sequence analysis revealed an Asp240Gly substitution. This substitution has already been reported in CTX-M-15, CTX-M-16, CTX-M-32 and CTX-M-27, and is known to confer high-level resistance to ceftazidime.

### Hospital versus community specimens

Among the 16 CTX-M-15-producing isolates, 2 isolates (12.5%) were reported to be from patients with community-acquired infections, which is uncommon. The two isolates were collected from urine specimens, while the remaining isolates were collected from hospitalized patients. The majority of the CTX-M-producing isolates were associated with urinary tract infections (11 strains), representing 69% of all isolates. Three (19%), one (6%) and one (6%) were isolated from blood, sputum and a nephrostomy swab, respectively.

### Plasmid and replicon type determination

S1-nuclease plasmid profiles were obtained for all CTX-M-producing isolates. A total of one to two plasmids, ranging in size from ~40 to 210 kb, were observed per strain, as shown in Table 1. Eight isolates produced a single plasmid, while two plasmids were identified from the other eight isolates. The putative high-molecular-weight plasmids of ~210 kb obtained were confirmed after several gel electrophoresis runs. Fingerprint analysis of extracted plasmids revealed six different restriction profiles using the EcoR1 endonuclease (Figure 1).

In this study, PCR analysis showed that the IncN plasmid was always present when the *blaCTX-M-15* gene was identified, even in the absence of IncFII. These results are the first to indicate the role of IncN replicon types in the dissemination of the *blaCTX-M-15* genes in *K. pneumoniae*, although an IncN plasmid has been found on one occasion to carry the CTX-M-15 gene in *E. coli*. It was confirmed that the mobilization and spread of CTX-M-15 seems to be related to IncFII plasmids. Similar to our results, *blaCTX-M-32* and *blaCTX-M-1* (CTX-M group 1) have been located on a single ~40 kb IncN plasmid or associated with an IncI1/M plasmid of 50 kb from different *E. coli* strains in Spain since 2000. Concern was raised regarding the dissemination of broad-host-range plasmids, such as IncN, because they contribute to the further spread of CTX-M-1-like enzymes among other members of Enterobacteriaceae.
Exploration of the regions surrounding bla_{CTX-M-15} genes

PCR identified the insertion sequence IS\textsubscript{Ecp1} upstream of the bla_{CTX-M-15} gene in all strains (Table 1) except one (strain 115). IS\textsubscript{Ecp1} was detected by sequencing 48 nucleotides upstream of bla_{CTX-M-15}. A 48 bp sequence, previously described for bla_{CTX-M-15} from India has previously been named as the W sequence.\textsuperscript{12,15,42} The insertion sequences IS\textsubscript{Ecp1} or IS\textsubscript{Ecp1}-like have been repeatedly identified upstream of many bla_{CTX-M} genes, and play an important role in the mobilization and expression of these genes.\textsuperscript{12,42,43}

Incidence of class 1 and class 2 integrons

Class 1 integrons are widespread genetic elements that allow usually promoterless bacteria to capture and express gene cassettes. These integrons contribute to the dissemination of antibiotic resistance genes between bacteria of the same or of different species.\textsuperscript{44} The results identified class 1 integrons in all but one of the CTX-M-producing \textit{K. pneumoniae} isolates (Table 1). PCR identified two different sizes of class 1 integrons, the largest one was \sim \textasciitilde \textasciitilde 2 kb and the second was \sim 1 kb in size. After sequencing of representative samples, the three different

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**Figure 1.** EcoR1 restriction endonuclease analysis of CTX-M-15-producing isolates. Lane 1, isolate 195; lane 2, isolate 115; lane 3, isolate 113; lane 4, isolate 215; lane 5, isolate MB; lane 6, isolate 187.

**Figure 2.** PFGE dendrogram of CTX-M-producing \textit{K. pneumoniae} isolates obtained after XbaI-digested the chromosomal DNA. A band position tolerance of 1\% was used in the PFGE-pattern analysis with the Dice band-based similarity coefficient.
gene cassette arrangements were shown to be as follows: dfrA12 +aadA2; aadA1; and aadA2. Similar to our study, class 1 integrons, including dfr and aadA gene cassettes, have been reported as the most prevalent type of integrons.\textsuperscript{45} The 3'-conserved segments of the class 1 integrons usually contain the genetic determinants qacE\textsubscript{A1} and sul\textsubscript{1}.\textsuperscript{66} The genetic organization and regions surrounding the bla\textsubscript{CTX-M-15} genes were investigated by PCR. The 15 integron-positive isolates did contain the conserved qacE\textsubscript{A1}-sul\textsubscript{1} region. All isolates were tested for the presence of class 2 integrons using int\textsubscript{I2} primers. Only two isolates were positive for class 2 integrons and these two isolates were positive for class 1 integrons as well.

Four of these isolates were negative for the integrase gene intI1. Interestingly, the isolates that were negative for 3'-conserved segments gave a band for the integrase gene intI1.

**Transfer of resistance**

Cefotaxime resistance transferred by transconjugation and transformation was obtained for all CTX-M-producing *K. pneumoniae* isolates.

**Epidemiological analysis**

PFGE analysis was used to analyse the molecular epidemiology of the 16 CTX-M-15-producing clinical isolates. As indicated from the result of the PFGE analysis (Figure 2), there were five different clones of CTX-M-15-producing isolates.

**Conclusions**

Despite the prevalence of CTX-M-15 in the UK, there have been few investigations on *Klebsiella* spp. with CTX-M enzymes. This work confirms the emergence of hospital- and community-acquired CTX-M-type enzymes in *K. pneumoniae* and their spread in Edinburgh, Scotland. Using PCR, all isolates were associated with IncFII or with the broad host range IncN plasmid. We identified five PFGE clusters of *K. pneumoniae* isolates conferring high-level resistance to most antibiotics tested.

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**Transparency declarations**

None to declare.

**References**


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