Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum β-lactamases in York, UK

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Objectives: This study was conducted to detect the presence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae within the faecal flora of both community- and hospital-based patients in York and to characterize the blaTEM, blaSHV and blaCTX-M genes present in these isolates.

Methods: One thousand faeces samples were collected and screened at York Hospital during October–December 2003. Ninety-five non-duplicate Enterobacteriaceae isolates resistant to third-generation cephalosporins were recovered; 22 isolates were selected for further study on the basis of a positive double disc diffusion test for ESBL production. Antibiotic susceptibility testing was performed to a range of antibiotics. The TEM, SHV and CTX-M genes were detected by PCR and the DNA sequenced.

Results: The distribution of ESBL-positive isolates from the hospital and community was 1.4:1. These included nine Escherichia coli, seven Enterobacter cloacae, four Citrobacter freundii and a single isolate each of Klebsiella spp. and Salmonella spp. A total of 17 isolates contained blaCTX-M (five blaCTX-M-15, three blaCTX-M-14 and nine blaCTX-M-9). ISEc1 was present in isolates expressing CTX-M-14 and -15, but was absent upstream of In60-associated blaCTX-M-9. E. coli isolates also contained either a blaTEM-1 or blaTEM-2, whereas six of the E. cloacae carried blaSHV-12 and the Klebsiella spp. blaSHV-36 in addition to blaCTX-M-9. The single Salmonella spp. carried blaSHV-12.

Conclusions: The overall prevalence of ESBL in isolates of Enterobacteriaceae from York was 1.9%. ESBL-producing isolates were found in both the community and hospital, with the CTX-M type most common. This is also the first report of an ESBL-producing Salmonella in the UK.

Keywords: ESBLs, Salmonella, Enterobacteriaceae

Introduction

The production of extended-spectrum β-lactamases (ESBLs) by Enterobacteriaceae has been documented since the introduction of third-generation cephalosporins (3GCs) into clinical usage. Whereas plasmid-mediated or hyperproduction of AmpC-type and other β-lactamases have been the most common cause of resistance to 3GCs, ESBL-producing Enterobacteriaceae have recently become more frequent in the UK.1–3 Currently, the ESBLs associated with 3C-resistant Enterobacteriaceae can be divided into the ‘big three’ families of TEM-, SHV- and CTX-M-type β-lactamases. TEM and SHV variants are reliant on key amino acid substitutions to increase their substrate profile to include the 3GCs, whereas the CTX-Ms have an intrinsic extended-spectrum profile.4 A national survey on the prevalence and mechanisms of resistance to 3GCs in clinically relevant Enterobacteriaceae in the UK was last conducted over a decade ago, where only TEM- and SHV-type ESBLs were screened for at a molecular level.5 The findings of this report gave an ESBL phenotype frequency of 1% of unselected isolates of Enterobacteriaceae from a wide range of locations.5 Since this time, we have seen the emergence and global dissemination of the CTX-M-type β-lactamases, which have become the predominant ESBL type in a number of Asian and South American countries.6–8 The recent reports of the emergence of CTX-M β-lactamases in the UK have come from hospital-based patients...
from areas with a large multicultural and transient population: Belfast, Newcastle, Leeds, Birmingham and London.\textsuperscript{2,3} Isolate samples have been predominantly recovered from the urinary tract, whereas the carriage rates of ESBL from faecal flora or community-based patients remains unknown. York is a city in Northern England with a less dynamic and ethnically diverse population of 181 000 (http://www.statistics.gov.uk/census2001/pyramids/pages/00ff.asp). The aim of this study was to detect the presence of ESBL-producing Enterobactiaceae within the faecal flora of both community- and hospital-based patients and to characterize the molecular type of TEM, SHV and CTX-M ESBLs present in this setting. We also revisited the collection of isolates obtained during 1990–1991 by Piddock and co-workers,\textsuperscript{5} ESBL present in this setting. We also revisited the collection of isolates obtained during 1990–1991 by Piddock and co-workers,\textsuperscript{5} ESBL present in this setting.

Materials and methods

Clinical strains

Faeces samples submitted for the diagnosis of diarrhoeal disease from community- and hospital-based patients in and around York were collected during October–December 2003 at York Hospital. One thousand consecutive non-duplicate faeces samples were screened; 565 from general practice (community) patients, 394 from hospital inpatients, 20 from hospital outpatients and 21 from old people’s homes/long-stay hospices. Samples were screened by inoculating them onto three separate plates of MacConkey agar containing vancomycin (6 mg/L) and either cefpodoxime (4 mg/L), cefotaxime (1 mg/L) or ceftazidime (1 mg/L). In total, 95 non-duplicate isolates of the family Enterobacteriaceae were recovered. Twenty-two isolates were selected for further study on the basis of an initial positive test for ESBL production by a double disc diffusion method using both cefotaxime and ceftazidime discs (DDD test).\textsuperscript{10} These included nine Escherichia coli, seven Enterobacter cloacae, four Citrobacter freundii, one Klebsiella spp. and one Salmonella spp. The isolates were identified by conventional biochemical methods\textsuperscript{11} and species identification of the isolates was achieved using API 20E test strips (bioMérieux S.A., Marcy l’Etoile, France). The 1990–1991 collection of 58 extended-spectrum cephalosporin-resistant E. coli (n = 29) and Klebsiella spp. (n = 29) isolates from UK hospitals were accessed for CTX-M screening.\textsuperscript{3}

Antibiotic susceptibility testing

The BSAC disc diffusion susceptibility test was performed on the 22 isolates on Iso-Sensitest agar to document associated antibiotic resistance markers (Oxoid, Basingstoke, UK). A disc diffusion method was used with the following antibiotics: co-amoxiclav, cefpodoxime, ceftazidime, cefotaxime, cefuroxime, piperacillin/tazobactam, cefepime, cefotixin, ceftriaxone, imipenem, ciprofloxacin, gentamicin and aztreonam.\textsuperscript{12} The minimum inhibitory concentration of cefpodoxime, ceftazidime and cefotaxime were determined using NCCLS methodology.\textsuperscript{13,14} For all 22 isolates, the E. coli strain ATCC 10418 and Klebsiella pneumoniae ATCC 700603 were used as antibiotic-susceptible and ESBL-positive controls, respectively.

RAPD typing of bacteria

Template DNA was prepared from bacteria grown overnight at 37°C on nutrient agar plates. Crude DNA extracts were obtained by suspending a colony in 50 μL of distilled water in an Eppendorf tube and boiling at 95°C for 5 min. Each RAPD PCR reaction consisted of a Ready-To-Go PCR bead (Amersham Biosciences, Amersham, UK), 25 pmol of either Eric1 primer (5’- ATG TAA GCT CCT GGG GAT TCA C-3’) for E. coli, or primer P1 (5’- GGT GCG GGA A-3’) for E. cloacae (Invitrogen, UK) and 2 μL of DNA suspension in a final volume of 25 μL. The amplification protocol was 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 25°C, 4 min at 72°C for E. coli and 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C for E. cloacae. RAPD products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 mg/L) with TAE running buffer. A molecular size marker ladder, Hyperladder 1, was included on all gels (Bioline, London, UK). Banding patterns were evaluated visually.

PCR amplification

All isolates were initially screened for the presence of bla\textsubscript{TEM}, bla\textsubscript{SHV} and bla\textsubscript{CTX-M} by a previously described multiplex PCR protocol,\textsuperscript{15} and subsequently whole ORF products were amplified as described previously.\textsuperscript{16} All isolates were further screened for the presence of bla\textsubscript{CTX-M} using a multiplex primer PCR containing 0.25 pmol of the universal forward primer IMA3 (5’- ATG TGA GYA CCA GTA ARG TRA TGG C –3’) and an equal mixture of primers cxr1 (5’-CAA ACC GTT GGT GAC GAT –3’), cxr2 (5’- CGG TGG GTT ACG ATT TTC GC-3’), cxr8 (5’-AAC CGT CCG TGA CRA TTY TS-3’) and cxr9 (5’- CCT TCG GCG ATG ATT CTC GC-3’), which are designed to give product sizes of 693 bp (CTX-M-1 group), 684 bp (CTX-M-2 group), 695 bp (CTX-M-8 group) and 683 bp (CTX-M-9 group).\textsuperscript{17} PCRamplification-positive isolates were further tested using individual pairs of primers to identify which CTX-M group the gene belonged to. For blac\textsubscript{TX-M} associated with ISEcp1, amplification of a ~1300 bp product containing the whole CTX-M-1 group ORF or Toho-2/CTX-M-9 group ORF was as described previously.\textsuperscript{18} The whole ORF of In60-associated blac\textsubscript{TX-M} was amplified using primers In60ctxL (5’-GAT ACT TCG GAT GAG GAG CA-3’) and In60ctxR (5’-GGA AAC AAT GAG AAA ACT GC-3’) designed from the Spanish In60-associated CTX-M-16.\textsuperscript{18} All primers were synthesized by Invitrogen (Invitrogen, UK). Bacterial DNA was prepared by suspension of one or two colonies in 50 μL of sterile distilled water and heating at 95°C for 5 min. PCR amplification was carried out using the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Nucleotide sequence determination

The sequence of the blac\textsubscript{TX-M}, blac\textsubscript{TEM} and blac\textsubscript{SHV} ORF PCR products were determined using a Big Dye PCR reaction (Applied Biosystems Inc, Foster City, CA, USA) and analysed on a primus HT 3700 DNA analyser by the functional Genomics Laboratory, University of Birmingham, UK.

Results

Bacterial strains

Initial disc diffusion susceptibility and RAPD-typing. Isolates presumptively identified as ESBL producers were subjected to susceptibility testing using the disc diffusion method (Table 1). Eight of the nine E. coli isolates and the Salmonella spp. were from community patients, the remaining 13 isolates were collected from hospital patients. RAPD was performed on isolates of E. coli and E. cloacae. Two of the nine E. coli strains shared
### Table 1. Summary of results

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Species</th>
<th>RAPD Clone</th>
<th>CTX-M type</th>
<th>TEM/SHV type</th>
<th>Mobile element</th>
<th>Disc test antibiotic resistance</th>
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<td>–</td>
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<td>–</td>
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<td>In60</td>
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H, hospital isolate; GP, community isolate; AMC, co-amoxiclav; CPD, cefpodoxime; CAZ, ceftazidine; CTX, cefotaxime; CXM, cefuroxime; TZP, piperacillin/tazobactam; FEP, cefepime; FOX, cefoxitin; CRO, ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin; ATM, aztreonam; CLA, clavulanic acid.
an identical RAPD pattern (clone D), which included one from a hospital isolate. Five of the seven E. cloacae isolates gave identical RAPD patterns and were considered to be the same clone (clone II). All remaining isolates showed different banding patterns by RAPD typing and were considered different strains.

Detection and identification of TEM, SHV and OXA-1 genes
Of the 22 isolates tested, the nine E. coli carried blaTEM and eight other isolates carried blaSHV. The remaining five isolates (four C. freundii, one E. cloacae) did not contain either blaTEM or blaSHV. No isolates contained blaOXA-1. Sequence analysis of the TEM ORF PCR products of the five strain A E. coli were identified as blaTEM-2, with the remaining four TEM PCR products identified as a blaTEM-1. The SHV PCR product of six of the E. cloacae and the Salmonella were identified as blaSHV-12, whereas the Klebsiella spp. carried a blaSHV-36. These results are summarized in Table 1.

Detection and identification of the CTX-M genes
PCR was performed on all isolates initially using a five primer mixture to detect the presence of blaCTX-M from all four known CTX-M family groups. None of the 58 isolates collected during 1990–1991 contained a blaCTX-M gene (data not shown). Seventeen of the 22 York strains were CTX-M positive (see Table 1) and were used in a further round of PCR reactions using primer pairs of fMA3, together with one of cxr1, cxr2, cxr8 or cxr9, which are specific for blaCTX-M-1, blaCTX-M-2, blaCTX-M-3 and blaCTX-M-9 groups of CTX-M, respectively. With fMA3 and cxr2 or cxr8 primers, no amplification products were detected from any isolates. Using primers fMA3 and cxr1, 693 bp amplification products were detected in five E. coli isolates, identical in size to the CTX-M-3 positive control. PCR amplification and subsequent sequencing of the whole ORF of an ~1300 bp product from these isolates confirmed the presence of a blaCTX-M-15, and associated ISecp1 element upstream. Using primers fMA3 and cxr9, 683 bp amplification products were detected in 12 isolates with a profile identical to the CTX-M-14 positive control used in these experiments. Only three isolates of E. coli gave PCR amplicons of ~1300 bp using ISecp1 and IS903 specific primers, which were then used for sequencing and confirmed as blaCTX-M-14. The ORF of the remaining isolate was amplified using primers specific to the flanking region of CTX-M-9 associated with In60, to give an amplicon identical to blaCTX-M-9 except for a silent mutation at base position 195 (T → C). Three C. freundii isolates did not produce TEM- SHV- or CTX-M-type ESBLs, one failing to show clavulanate enhancement, although two isolates—Y16 and 20—showed enhancement with cefotaxime but not ceftazidime.

Disc diffusion susceptibility of ESBL-carrying isolates
The antibiogram (13 antibiotics) was determined for the 22 DDD test-positive Enterobacteriaceae isolates from York (Table 1). All isolates were resistant (using BSAC criteria) to cepodoxime, cefotaxime, ceftriaxone and piperacillin/tazobactam where either/or blaSHV/CTX-M was detected. Of these isolates, only the single Salmonella spp. isolate was susceptible to cefepime and aztreonam. The three CTX-M-14-producing E. coli, one CTX-M-9-producing E. cloacae and the Klebsiella spp. were susceptible to ceftazidime, which correlated with MIC data. All isolates were susceptible to imipenem, and for three E. cloacae and one E. coli this was the only antibiotic tested to which these isolates were susceptible. Six of the nine E. coli isolates were resistant to ciprofloxacin. Although the number of isolates is low, this is more than would be expected from UK surveillance data for E. coli. All of the E. coli isolates producing CTX-M-15 were resistant to ciprofloxacin.

Discussion
This report offers an insight into the current prevalence and molecular types of ESBL-producing organisms in York in both the hospital and community setting.

A previous survey of the prevalence and types of ESBLs produced by non-selected Enterobacteriaceae in UK hospitals from 1990–1991 found a frequency of 1%, with the most commonly characterized ESBLs being the SHV-type. This older study indicated that a number of isolates contained another non-TEM/SHV ESBL type, raising the possibility that blaCTX-M may have been present in the UK at that time. Further testing during the study reported here failed to find blaCTX-M.

Given the prevalence and pattern of ESBL types in 1991 and the recent appearance of CTX-M-type β-lactamases in the UK, it is surprising to find the number and variety of CTX-Ms produced by our isolates from York. The rapid emergence of the CTX-Ms as the predominant ESBL type is not an isolated phenomenon. A recent report on ESBL types in Enterobacteriaceae in Argentinean public hospitals found that CTX-Ms accounted for roughly two-thirds of all ESBLs found, with similar findings in studies in Japan, China, Taiwan and Spain.

This is in contrast to studies of isolates from the early 1990s, which demonstrated the absence of CTX-M-type β-lactamases and the prevalence, for instance, of SHV-12 and -15 in the Far East. Whereas we found blaCTX-M-12 and blaCTX-M-15-carrying clones of E. cloacae and E. coli, respectively, the success of these clones alone does not account for all blaCTX-M found. The recent detection of distinct strains of E. coli carrying blaCTX-M-15 in different locations in the UK further supports our findings.

The blaCTX-M genes are associated with one of two types of mobile element, most commonly insertion sequences, such as ISecp1 as seen with our CTX-M-14- and CTX-M-15-producing isolates, or integron-associated blaCTX-M (e.g. In60), as seen with our blaCTX-M-9-carrying strains. To our knowledge, this is the first occasion that blaCTX-M have been found associated with both types of mobile elements in one collection, and suggests an indication of further mixing and global dissemination of these genes and associated mobile genetic elements.

In our study, two different ESBL SHV-types were found, SHV-12 and SHV-36. Only the one Klebsiella spp. from York carried blaSHV-36, a new SHV type found previously in a K. pneumoniae isolate from Leeds, which is 20 miles from York (GenBank accession no. AF467947). All bar one of the seven blaSHV-12 carrying E. cloacae also carried blaCTX-M-9. This co-carriage of ESBL genes is not a novel finding but is uncommon. In the report of the first description of blaCTX-M-14 and blaCTX-M-13, these genes were carried in five of 12 CTX-M-positive isolates of E. coli, K. pneumoniae and E. cloacae, together with either blaSHV-12 or blaSHV-11. These isolates were found during 1997/8 in Guangzhou, China, from a surveillance study, during which time ESBL rates doubled over a year to 33%
and 37% for *E. coli* and *K. pneumoniae*, respectively.

In the recent national study of ESBL-producing Enterobacteriaceae isolates from Canada, a single clinical strain harbouring the CTX-M-14 gene was found to also contain an SHV-12. The International Klebsiella study group also found co-carriage of CTX-M-2 and SHV-5 genes in single isolates from Argentina and Turkey.

The clinical significance and epidemiological impact of these findings is yet to be ascertained.

The *Salmonella* species produced SHV-12, and whereas a variety of SHV-type ESBL-producing *Salmonella* strains have been reported from Canada and Poland (SHV-2a), Romania (SHV-5) and Italy (SHV-12),

we believe this to be the first reported case of an ESBL-producing *Salmonella* isolate in the UK. Interestingly, CTX-M ESBLs of types 2, 3, 4 and 9 have been reported in *Salmonella* isolates for a while from South America and Europe.

The recent report of CTX-M-14 in *Salmonella enteritidis*, which probably involved in vivo transfer of a plasmid carrying *bla*<sub>CTX-M-14</sub> from an *E. coli* strain in the faeces of the patient, suggests that CTX-M-type ESBL-producing *Salmonella* may become more common in Europe.

In this study, we tested isolates for TEM-, SHV- and CTX-M-type genes. Whereas these genes were detected in 19 isolates, accounting for their ESBL phenotype, other ESBLs such as PER-1 or VEB-1 cannot be excluded without further testing, but were beyond this study. One of the *C. freundii* isolates carried a *bla*<sub>CTX-M-9</sub>, but the remaining three isolates selected by disc diffusion testing did not. Further testing of these isolates for AmpC-type β-lactamases and other mechanisms may elucidate how resistance is conferred in these isolates. The hospital antibiotic policy results in cefotaxime and ceftriaxone being the biotic policy results in cefotaxime and ceftriaxone being the

select for cefotaximases such as CTX-M-14.

The majority of studies of ESBL-producing Enterobacteriaceae have looked at hospital isolates, therefore the occurrence of these enzymes in community isolates from York is of interest. To comply with ethics committee rules, we could not obtain information to rule out completely prior hospitalization in all of the community patients. A Spanish study examining ESBL production in *E. coli* and *K. pneumoniae* involved in urinary tract infection found seven of the 1580 (0.4%) *E. coli* strains tested to be ESBL positive, and suggested a link to previous hospitalization. A number of risk factors have been identified as linked with the acquisition of community-acquired infections involving ESBL-positive isolates. These are previous hospitalization or antibiotic therapy within the previous 3 months, old age (>60 years), male gender, confinement to bed with debilitation and urinary catheterization.

Thirty-nine of 2599 (1.5%) Enterobacteriaceae were ESBL positive in a study conducted in the Aquitaine region of France, and demonstrated a variety of TEM, SHV and CTX-M-type enzymes involved with co-existence of different β-lactamase genes within the same isolate. This is similar to our findings from York, with strain and/or plasmid dissemination in private healthcare centres identified as possible sources for outbreaks of ESBL-producing bacteria.

In conclusion, our data suggest that ESBLs in both hospital and community settings are capable of persistence, that these genes are present in at least some true community isolates and that their incidence may be rising. A range of CTX-M-type ESBLs and dissemination of these genes into a number of Enterobacteriaceae by a variety of mechanisms of horizontal gene transfer were found. This demonstrates the need to monitor both hospitalized and general practice patients for the further emergence of transferable resistance to extended-spectrum cephalosporins.

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References

CTX-M β-lactamases in York, UK


