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Regional survey of CTX-M-type extended-spectrum β-lactamases among Enterobacteriaceae reveals marked heterogeneity in the distribution of the ST131 clone

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Objectives: To establish the prevalence and diversity of clinically significant extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae harbouring $bla_{CTX\text{-}M}$ in the West Midlands region of the UK.

Methods: During a 2 month period, 370 consecutive, non-duplicate isolates were collected from 13 laboratories. Isolates were screened for the presence of $bla_{CTX\text{-}M}$ by multiplex PCR and genotyped using denaturing HPLC (DHPLC). Clonal relationships were studied by PFGE and O25b-ST131 *Escherichia coli* were identified by PCR.

Results: Two hundred and ninety-four out of 345 ESBL-producing isolates (85.2%) carried $bla_{CTX\text{-}M}$. CTX-M group 1 enzymes were expressed in 284 (96.6%) isolates, with the other 10 carrying group 9, 2 and 25/26 genes. All group 1 isolates had $bla_{CTX\text{-}M\text{-}15}$ DHPLC profiles. The $bla_{CTX\text{-}M}$ *E. coli* were split into 23 PFGE clusters. The largest cluster (RE1) was indistinguishable from the previously described strain A and all but one harboured $bla_{CTX\text{-}M\text{-}15}$. A total of 66% of *E. coli* were O25b-ST131 positive.

Conclusions: The CTX-M-15-producing RE1 clone (strain A) is the predominant clone in the West Midlands. This clone has spread throughout the region since its emergence in an outbreak 3 years earlier. Most, but not all, RE1 isolates belong to the O25b-ST131 lineage, providing further evidence that this lineage plays a pivotal role in the clonal dispersal of CTX-M-15-producing Enterobacteriaceae. Strain A was found to be considerably more heterogeneous than when first described and has acquired greater resistance to gentamicin. Approximately one-third of CTX-M producers represented a wide variety of unrelated strains. The study shows the rapid spread and diversification of CTX-M-producing Enterobacteriaceae over a 3 year period.

Keywords: ESBLs, molecular epidemiology, O25b-ST131

Introduction

The West Midlands region of the UK covers an area of ~13 000 km^2, encompassing both large conurbations and remote rural areas, and has a population of ~5.4 million. In 2003, a major outbreak of CTX-M-15-type, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* belonging to the O25b-ST131 clone, and designated epidemic strain A by PFGE, was identified in Shrewsbury in the north-west of the region.[1] Conducted 3 years after the outbreak, this study aimed to establish the prevalence of this clone and other CTX-M-harbouring strains of Enterobacteriaceae in the West Midlands region using a structured survey. The study was carried out prospectively over a period of 2 months and included all new isolates of ESBL-producing Enterobacteriaceae from both community and hospitalized patients from the 13 major hospitals within the region. Isolates were fully characterized with respect to $bla_{CTX\text{-}M}$, and two molecular typing methods, PFGE and PCR duplex detection of *E. coli* clone O25b-ST131, were used to assess the extent of clonality within CTX-M-producing isolates. This enabled the identification of possible shifts in the predominance of major clones and the emergence of new epidemic clones.

Methods

Bacterial isolates, ESBL screening and antimicrobial susceptibility testing

Between April and May 2006, a total of 370 consecutive, non-duplicate isolates of Enterobacteriaceae, already identified as ESBL-producing

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organisms by local laboratory criteria, were collected from 13 hospitals in the West Midlands region of the UK. These isolates were obtained from both community and hospitalized patients. Community-acquired isolates were defined as those from patients who had not been hospitalized in the preceding 3 months; hospitalized patients whose first positive culture had been obtained >48 h after hospital admission were considered to have nosocomial infections. Organism identity was confirmed using API 20E (bioMérieux, Marcy-l'Étoile, France). All isolates were screened for susceptibility to cefpodoxime by the BSAC disc diffusion method (http://www.bsac.org.uk/Resources/BSAC/Ecoliklebsiella.pdf). ESBL production was confirmed phenotypically using cefpodoxime susceptibility, followed by ceftazidime and cefpirome ± clavulanic acid double-disc diffusion. Antimicrobial susceptibility to a range of antimicrobial agents was also determined according to BSAC guidelines. Isolates were regarded as being multidrug resistant if they had reduced susceptibility to at least three structurally unrelated antibiotics.

PCR amplification

Confirmed ESBL-producing isolates were screened for the presence of bla_{CTX-M} by our previously described multiplex PCR protocol.[2] Four sets of primers were used to amplify fragments of bla_{CTX-M} open reading frames of CTX-M-type ESBLs, designed to give product sizes of 341 bp (group 2), 293 bp (group 9), 255 bp (group 1) and 207 bp (groups 25/26 and 8). Detection of insertion sequence (IS) IS*26* was performed for all isolates carrying bla_{CTX-M} using primers IS*26*F and IS*26*R, described previously.[1]

Denaturing HPLC (DHPLC) and sequencing

The bla_{CTX-M}-positive isolates were further genotyped using the DHPLC method previously published by our group.[3] Representative control strains GZ3 (CTX-M-3), Y19 (CTX-M-9), J1 (Toho-1) and ESBL530 (CTX-M-25) were used as reference standards for groups 1, 9, 2 and 25/26, respectively. The chromatographic signatures of unknown bla_{CTX-M} types were compared with the reference peaks. DHPLC genotyping results were confirmed by DNA sequencing of a limited number of isolates (30) from the study. PCR fragments covering the whole open reading frame of the relevant bla_{CTX-M} gene were cleaned by using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The PCR product was sequenced using an ABI BigDye Terminator (version 3.0) cycle sequencing kit and the sequences were analysed on an ABI 3700 DNA Analyzer.

PFGE and identification of the O25b-ST131 E. coli clone by PCR

All bla_{CTX-M}-positive *E. coli* and *Klebsiella* isolates were typed by PFGE (Bio-Rad Laboratories, Hemel Hempstead, UK) after the digestion of genomic DNA with XbaI. The PFGE banding patterns were analysed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The unweighted pair-group method using arithmetic averages was used to obtain a dendrogram. Isolates were considered clonally related if the Dice coefficient correlation was ≥85%. The duplex PCR method recently described by Clermont *et al.*[4] was employed to identify the O25b-ST131 *E. coli* clone using PCR primers O25pabBspe.F, O25pabBspe.R, trpA.F and trpA2.R.

Results

Detection of bla_{CTX-M} and IS elements, demographics, and epidemiology

Three hundred and forty-five (93.2%) of the 370 isolates submitted were confirmed as ESBL producers. Multiplex PCR assay revealed that 294 (85.2%) of these carried bla_{CTX-M}. The 294 bla_{CTX-M}-positive isolates included 232 *E. coli*, 58 *Klebsiella* spp., 3 *Enterobacter* spp. and 1 *Proteus vulgaris*. One hundred and seventy-five (59.5%) originated from hospital patients and 119 (40.5%) originated from the community. The majority of these isolates were associated with urinary tract infections (241, 82.0%), but there were also 25 blood culture isolates (8.5%).

PCR amplification identified IS*26* in 62 of the 294 (21%) bla_{CTX-M} producers, with two fragments of 400 bp (43/62) and 873 bp (19/62). Two of each type of IS*26* fragment were sequenced and no T to C change was found in the spacer region between IS*Ecp1* and $bla_{CTX-M-15}$ (GenBank accession numbers GU732831 and GU732832).[1] The distribution of the two fragment types for *E. coli* is shown in Table 1. Of the 58 *Klebsiella* spp., two produced the 400 bp fragment and six produced the 873 bp fragment.

Table 1. Clinical and molecular characteristics of all 232 bla_{CTX-M}-producing *E. coli* strains belonging to clone O25b-ST131 and non-O25b-ST131

	No. of isolates (%)	
Parameter	O25b-ST131, *n* = 154	non-O25b-ST131, *n* = 78
Specimen origin		
hospital	88 (57.1)	40 (51.3)
community	66 (42.9)	38 (48.7)
Specimen type		
blood	14 (9.0)	5 (6.4)
urine	126 (81.8)	68 (87.2)
others	5 (3.2)	5 (6.4)
Antimicrobial resistance		
ciprofloxacin	152 (98.7)	65 (83.3)
trimethoprim	142 (92.2)	67 (85.9)
gentamicin	75 (48.7)	43 (55.1)
bla_{CTX-M} genotype		
$bla_{CTX-M-15}$	153 (99.4)	71 (91.0)
$bla_{CTX-M-9}$	1 (0.6)	1 (1.3)
$bla_{CTX-M-14}$	0 (0)	4 (5.1)
$bla_{CTX-M-2}$	0 (0)	2 (2.6)
Presence of IS*26* element		
400 bp	41 (26.6)	0 (0)
873 bp	12 (7.8)	1 (1.3)
negative	102 (66.2)	77 (98.7)

Antimicrobial resistance pattern of bla_{CTX-M}-producing isolates

Some 82% of isolates proved to be multidrug resistant. Of these, 93.4% were resistant to ciprofloxacin, 91.4% to trimethoprim and 52.4% to gentamicin. Over half of all isolates were resistant to all three antibiotics. In addition, nine isolates showed reduced susceptibility to carbapenems.

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Molecular typing of bla$_{CTX-M}$-producing isolates by DHPLC

Of the 294 *bla*$_{CTX-M}$-producing isolates, 284 (96.6%) possessed genes encoding group 1 CTX-M enzymes and 6 harboured a *bla*$_{CTX-M}$ group 9 gene. Two *bla*$_{CTX-M}$ group 2 and two group 25/26 producers were also identified. DHPLC genotyping of group 1 isolates showed that all had *bla*$_{CTX-M-15}$ profiles. Specific chromatographic signatures of *bla*$_{CTX-M}$ were also obtained for the group 9, 2 and 25/26 isolates (four *bla*$_{CTX-M-14}$, two *bla*$_{CTX-M-9}$, two *bla*$_{CTX-M-2}$ and two *bla*$_{CTX-M-26}$). DNA sequencing was performed on 30 (10%) representative DHPLC-typed strains producing *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-26}$. Isolates were confirmed to possess *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-2}$, and three of four *bla*$_{CTX-M-14}$ producers had sequences identical to those in GenBank. However, one CTX-M-14 enzyme had three silent mutations at nucleotide positions 372, 570 and 702, with an A to G change at position 372, and G to A substitution at positions 570 and 702 (GenBank accession number GU732835).

Clonality characterization of bla$_{CTX-M}$ producers by PFGE analysis and O25b-ST131 E. coli clone detection

Molecular typing by PFGE was performed for both *bla*$_{CTX-M}$-producing *E. coli* and *Klebsiella* spp. PFGE banding patterns were obtained for 225 of the 232 *E. coli* and 52 of the 58 *Klebsiella* spp. isolates. A cluster was designated when isolates showed ≥85% similarity to PFGE profiles. These clusters consisted of 2–102 isolates. PFGE analysis revealed that 213/225 (95%) *bla*$_{CTX-M}$-positive *E. coli* fell into 23 clusters (seven of them were found in at least five isolates) and were defined as clones designated arbitrarily as RE1–23 and the seven major clusters (RE1–7) are shown in Figure 1. They were distributed between both hospital and community isolates [55% (118/213) and 45% (95/213), respectively]. The remaining 12 *E. coli* isolates presented unique banding patterns. Out of the 213 strains in these 23 clusters, 102 (48%) strains constituted the largest clone RE1 and shared the UK epidemic strain A PFGE profile; they were widely dispersed in 12 out of the 13 hospitals studied and all harboured *bla*$_{CTX-M-15}$, except for one, which carried *bla*$_{CTX-M-9}$. The second largest clonal group, RE2, accounted for 14.6% of the strains (31/213), of which 24 (77%) were hospital isolates. Clones RE16, 18 and 23 were each found at a single hospital.

Duplex PCR on all 232 *E. coli* isolates revealed that 66% (154/232) were O25b-ST131 positive. Of these, all but two were resistant to ciprofloxacin; resistance to trimethoprim and gentamicin was found in 92.2% and 48.7%, respectively. The O25b-ST131 isolates were represented in 14 of the 23 PFGE clusters (RE1–6, 8, 10, 12, 13, 16, 20, 22 and 23) and in two unique PFGE types (Figure 1). The majority of the strains in clones RE1 (99/102) and RE2 (25/31) were O25b-ST131 positive. In contrast, 9/10 isolates in the third largest clone, RE3, and 10/12 isolates with unique banding patterns were non-O25b-ST131. Of the eight isolates that did not harbour *bla*$_{CTX-M-15}$ (four CTX-M-14, two CTX-M-9 and two CTX-M-2), only one CTX-M-9 producer belonged to O25b-ST131. The clinical and molecular characteristics of O25b-ST131 and non-O25b-ST131 isolates, including *bla*$_{CTX-M}$ and IS*26* genotyping, are summarized in Table 1. The geographical distribution of RE1 and O25b-ST131 clones is shown in Figure 2.

PFGE analysis of 52 of the 58 typeable *Klebsiella* spp. identified 11 clusters (RK1–11) and a further 12 unique PFGE profile types. RK1 and RK2 included isolates from 5 out of 10 hospitals and represented 33% of the isolates; all were CTX-M-15 producers, apart from two strains, which harboured *bla*$_{CTX-M-26}$ and originated from the same hospital.

Discussion

By the end of 2003, CTX-M ESBLs had become the foremost cause of resistance to third-generation cephalosporins in Enterobacteriaceae in all regions of the UK.[5] In comparison with the South-East regional survey of 2004, where CTX-M types accounted for 51% and 82% of ESBLs in *E. coli* and *Klebsiella* spp., respectively,[6] we found a significantly higher prevalence of *bla*$_{CTX-M}$ [89% (232/260) and 88% (58/66) for *E. coli* and *Klebsiella* spp., respectively]. This is consistent with recent reports from Ireland (89%),[7] China (89%),[8] Spain (86%)[9] and France (83%),[10] with the highest prevalence (96.6%) being documented in Thailand.[11]

The distribution of predominant *bla*$_{CTX-M}$ genotypes varies geographically.[12–15] CTX-M-15 is the dominant genotype worldwide,[16–18] with the exception of South-East Asia, where CTX-M-14 is common.[19] In a national survey in 2004, Woodford *et al.*[1] identified an epidemic CTX-M-producing strain (strain A), which had caused a major outbreak in Shropshire, part of the West Midlands region. This survey, which included 42 centres nationally, found that strain A was confined to London and the South-East, Northern Ireland and the West Midlands, with 61.4% of all isolates originating from the single West Midlands site at the centre of the outbreak.[1] Our survey is the first prospective study to characterize all *bla*$_{CTX-M}$-producing ESBL isolates in the West Midlands region. It was both large scale (294 CTX-M-producing isolates) and geographically comprehensive, reflecting the population distribution both in urban and rural areas (13 hospital laboratories).

Genotyping *bla*$_{CTX-M}$ by DHPLC revealed that CTX-M-15 is now the predominant CTX-M genotype across the region, providing evidence of its exceptionally successful dissemination in both community and hospital settings. The worldwide spread of *bla*$_{CTX-M-15}$-producing *E. coli* has been ascribed to the clonal expansion of a particularly virulent multidrug-resistant clonal group, O25b-ST131.[9,16,17] Indeed, all five major UK CTX-M-producing *E. coli* strains, including epidemic strain A identified in the 2004 national survey,[1] have been shown to be of the O25b-ST131 lineage.[20] Our study, carried out 3 years after the UK national survey,[1] revealed that *E. coli* isolates harbouring *bla*$_{CTX-M-15}$ were present across the West Midlands region and were mainly clonal; the O25b-ST131 clone accounting for 66% of all the *bla*$_{CTX-M}$-producing *E. coli* isolates (Table 1). The finding that nearly half of O25b-ST131 isolates were community acquired reinforces the suggestion that community-healthcare facilities could be a significant reservoir for this clone.[9] Significantly, almost all strains in the two largest PFGE clusters were members of the O25b-ST131 clonal group. The PFGE RE1 clone is indistinguishable from UK strain A, which caused a major outbreak in Shropshire in 2003. Despite the adoption of rigorous control measures,[5] our survey has shown that

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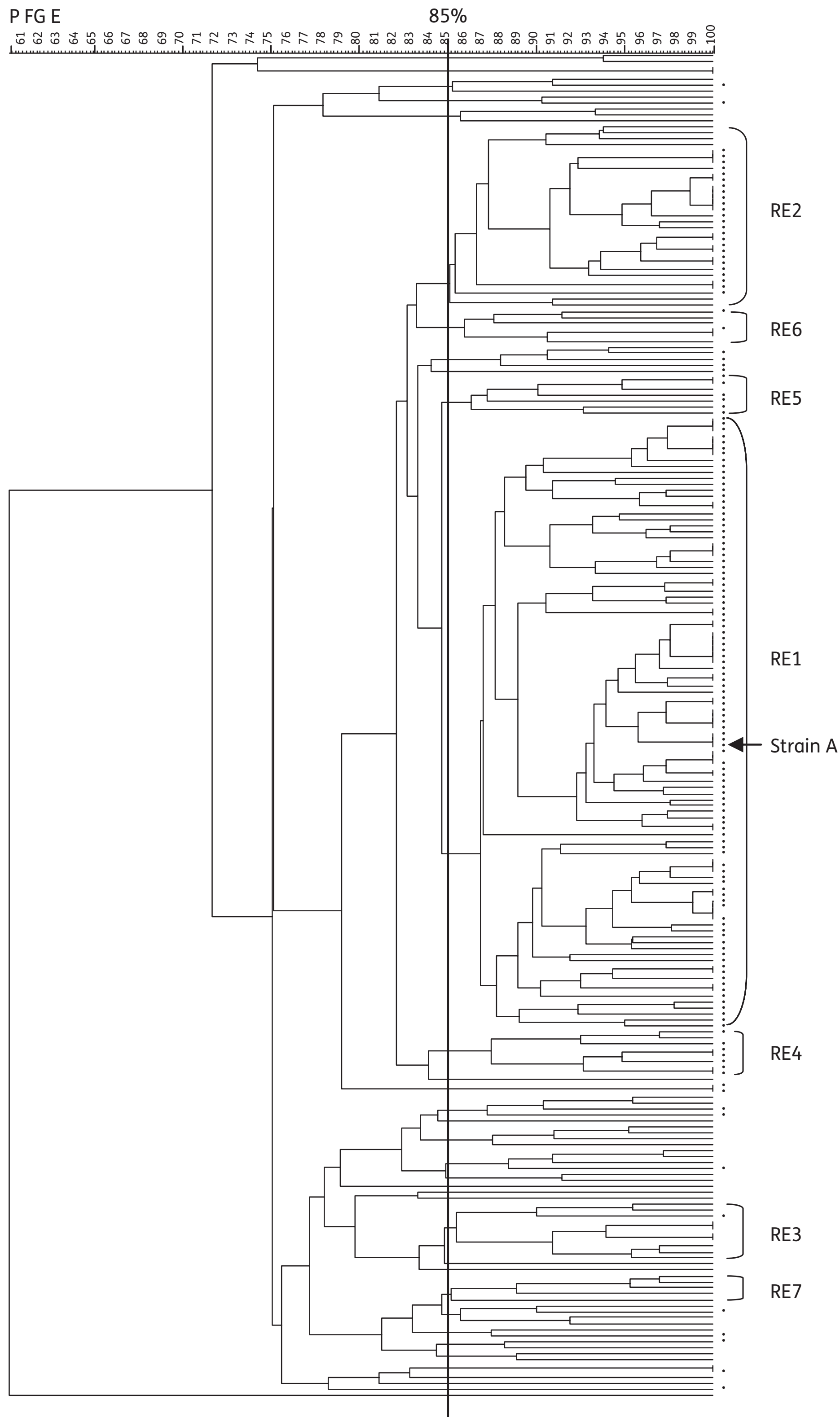

Figure 1. Dendrogram of PFGE patterns showing the genetic relatedness of 225 bla_{CTX-M}-harbouring *E. coli* isolates from 13 hospitals in the West Midlands region, UK. The O25b-ST131 isolates are indicated by dots. Seven major PFGE clusters (RE1–7) were identified with ≥85% similarity, which is marked by the vertical line. The arrow indicates the UK epidemic strain A, the reference strain used in this study.

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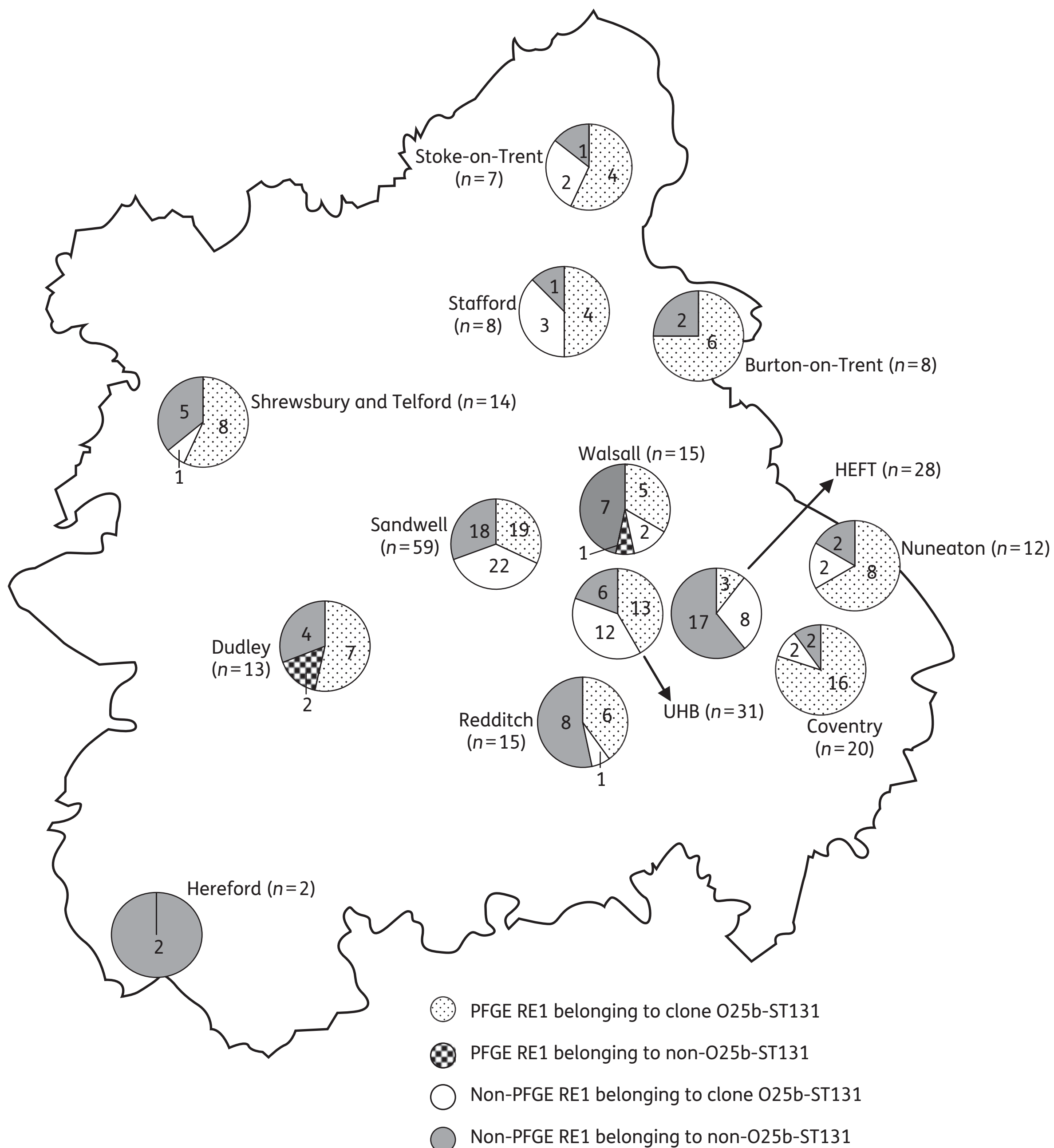

Figure 2. Geographical distribution and properties of bla_{CTX-M}-positive *E. coli* strains in the West Midlands region, UK. The total number (*n*) of bla_{CTX-M}-producing strains is indicated in parentheses. Heart of England NHS Foundation trust (HEFT) comprises three hospitals covering East Birmingham, Solihull and Sutton Coldfield. University Hospital Birmingham NHS Foundation Trust (UHB) comprises two hospitals covering Central and South Birmingham.

the strain, which emerged at the beginning of 2003, not only persisted in Shropshire, but also spread to all but one (the most geographically remote) of the 13 other regional hospitals. Nevertheless, between these hospitals, prevalence varied markedly (10%–80%), even within a single conurbation (Figure 2). The reasons for this epidemiological variation are unknown, but may be related to population demographics.

Woodford *et al.*[1] demonstrated the universal insertion of a copy of IS*26* into the IS*Ecp1* of plasmids carried by all strain A isolates, which yielded a 400 bp product in a PCR assay. In our study, only 37.3% (38/102) of strain A isolates produced a 400 bp IS*26* fragment. We also found 3/130 non-strain A isolates to be positive for a 400 bp fragment. The IS*26* 400 bp fragment was found only in the O25b-ST131 lineage, but the carriage rate of that type of plasmid was much lower than previously reported.[21] This suggests that O25b-ST131 carries a more diverse collection of plasmids than can be assumed from the earlier study.

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It was also reported by Woodford *et al.*[1] that four non-strain A isolates from a single centre with related PFGE patterns produced an IS*26* fragment of ~800 bp. We found 13 such *E. coli* strains, all but one of which belonged to the O25b-ST131 clone. Nine of these came from plasmids carried by strain A isolates and four were from non-strain A isolates. Of note, the isolates with an 873 bp IS*26* product were from seven different centres across the region. These data suggest that plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ are more diverse than previously thought. Interestingly, the 873 bp IS*26*-$bla_{CTX\text{-}M\text{-}15/\text{-}14}$ module has also been reported in India[21] (which has strong cultural links with the West Midlands) and Russia (GenBank accession number GQ385314). We found a similar configuration in six of our *Klebsiella* spp. There were only two previous reports in GenBank of the same configuration in *Klebsiella* spp. from Spain (GenBank accession number GQ845085) and Russia (GQ385315-7), suggesting that this arrangement may be involved in the mobilization of $bla_{CTX\text{-}M}$ into multiple species of Enterobacteriaceae.

In addition to the shared PFGE patterns, common serogroups and multilocus sequence typing (MLST) types, UK epidemic strain A was shown to be ciprofloxacin and trimethoprim resistant, but susceptible to gentamicin in accordance with the findings of Blanco *et al.*[9] from Spain. However, in our study of RE1 (strain A) clone isolates, susceptibility to gentamicin was seen in only 63.7%, despite co-resistance to ciprofloxacin and trimethoprim. A higher prevalence of resistance to gentamicin has also been described in Canada (86%)[22] and several geographically distant countries (50%).[16] Interestingly, all eight *E. coli* isolates harbouring non-$bla_{CTX\text{-}M\text{-}15}$ were susceptible to gentamicin.

Recently, attention has been drawn to the O25b-ST131 clonal group, primarily due to its vital role in the clonal dispersal of CTX-M-15-producing ESBLs across the world.[16] O25b-ST131 exhibits a significantly more extensive virulence profile than those of comparably resistant non-clonal group isolates[16,23] and its relative fitness may have contributed to its huge epidemiological success worldwide. Our study provides novel insights into the epidemiology of the dissemination and persistence of $bla_{CTX\text{-}M\text{-}15}$-producing O25b-ST131 in the West Midlands region of the UK. Whilst 154/232 $bla_{CTX\text{-}M\text{-}15}$-producing *E. coli* belonged to O25b-ST131, a significant number (78) of *E. coli* did not, and were represented by all but one of the 23 PFGE types and 10/12 unique profiles. This suggests that *E. coli* strains harbouring $bla_{CTX\text{-}M\text{-}15}$ that are not part of the widespread O25b-ST131 clone are also capable of dissemination and persistence in patients.

In summary, our survey shows for the first time the great diversity of CTX-M-producing strains of Enterobacteriaceae that has developed within the West Midlands region of the UK within a 3 year period. While the predominant CTX-M-15-producing RE1 clone (strain A) has persisted for >3 years after its emergence and has spread throughout the region, not all isolates are of the O25b-ST131 lineage and not all possess IS*26*. In addition, this strain now shows increased resistance to gentamicin. The majority of RE1 strains do belong to the O25b-ST131 lineage, but this clone is also represented in 13 other PFGE types identified in the study, providing further evidence of its pivotal role in the dispersal of CTX-M-15-producing ESBLs. There was remarkable heterogeneity amongst the CTX-M-producing isolates that were neither strain A nor of the O25b-ST131 lineage, demonstrating the continuing potential for genetic diversification and emergence of new epidemic strains. While O25b-ST131 strains were more likely to originate in the community than in hospital (60:40), other strains were evenly distributed between the two. Interestingly, the CTX-M-26-producing strain of *Klebsiella pneumoniae* responsible for an outbreak in Birmingham in 2001 was not detected in this study.[24]

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Transparency declarations

None to declare.

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