Genotypic Identification of Extended-Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective

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Abstract

Objectives: Urinary Tract Infections (UTIs) are one of the most common infections diagnosed in the United Kingdom (UK). The prevalence of Extended-Spectrum β-Lactamase (ESBL) producing UTIs has dramatically risen, limiting treatment options. The emergence and spread of ESBLs is thought to be through the horizontal transmission of antibiotic resistance plasmids IncL/M, IncFIA, IncFII and IncI1. These conjugative plasmids have been described as important vectors and directly linked to major outbreaks of antibiotic resistance. This study aimed to investigate the prevalence of ESBLs in Leicestershire, UK and their relationship with antibiotic resistance plasmids.

Methods: 236 ESBL producing uropathogenic Enterobacteriaceae isolates were obtained from the Leicester Royal Infirmary (Leicestershire, UK). ESBL production was confirmed phenotypically via the MAST ID double disc synergy test. ESBL-producing genes (CTX-M, SHV, TEM and OXA) were identified by multiplex PCR. The CTX-M family was then further characterised into (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) by multiplex PCR. The relationship between ESBL-producing genes and plasmid type was then investigated by multiplex PCR-based replicon typing to detect IncFIA, IncI1, IncL/M, IncN and IncFlII.

Results: ESBL genes were identified as follows: CTX-M (71.6%), OXA (7.6%), TEM (3.8%) and SHV (3.8%). Multiple resistance genes were detected in 16% of isolates. CTX-M genes were identified as follows: CTX-M-1 (84.1%), CTX-M-9 (12.5%), CTX-M-25 (1.7%), CTX-M-8 (1.1%) and CTX-M-2 (0.6%). Replicon typing results were as follows: IncL/M (29.2%), IncN (14.4%), IncI1 (5.1%), IncFlII (27.5%) and IncFIA (23.3%). A combination of IncL/M, IncFlII and IncFIA was the most common at 9.8%. A positive correlation between CTX-M and all plasmids except IncI1 was found.

Conclusion: CTX-M harbouring Enterobacteriaceae are associated with multiple plasmids, which can be linked to its rapid spread across the world. Prevalence studies help to inform policy about antibiotic stewardship and resistance evolution, aiming to reduce resistance levels in the future.

Keywords: Extended-spectrum-β-lactamases; Multiplex PCR; Antibiotic resistance; CTX-M; Urinary tract infection; Replicon typing; One health approach

Introduction

Urinary Tract Infections (UTIs) are one of the most common bacterial infectious diseases diagnosed in outpatients in the UK. Of the causes of UTIs, Uropathogenic Escherichia coli (UPEC) are the most prevalent [1]. Risk factors for acquiring a UTI include previous exposure to 3rd and 4th generation cephalosporins and fluoroquinolones, hospitalisation, old age, female gender, recurrent UTI infection, diabetes and catheterisation [1].

The most common type of resistance in UTIs is β-lactamase production, resulting in extended-spectrum β-lactamase (ESBL) producing bacteria [2]. These bacteria produce enzymes that can hydrolyse oxyimino-cephalosporins (ceftriaxone, cefotaxime, cefazidime and cefepime) and monobactams (aztreonam) but not cephamycins and carbapenens. Based on their amino acid sequence, these enzymes have been classed into four groups-A-D [2]. Class A enzymes have been shown to be the most clinically important, due to their link to treatment failure, increased morbidity, mortality and healthcare costs. In contrast to the class A, C and D serine β-lactamases, class B are metallo-β-lactamases. Class C β-lactamases are AmpCs, conferring resistance to not only third-generation cephalosporins but also β-lactam/β-lactamase inhibitor combinations. The most important class D β-lactamase is the OXA-type. There are 3 main families of class A: TEM, SHV and CTX-M while TEM, SHV and OXA ESBLs arise via substitutions in strategically positioned amino acids from the natural narrow-spectrum TEM-1/-2, SHV-1 and OXA-10 β-lactamases, all CTX-M variants demonstrate an ESBL phenotype [2]. Previously, most ESBLs detected were of the TEM/SHV group. From the 1990’s the CTX-M family became increasingly more common, and now it is reported to be the most prevalent type of ESBL detected at present worldwide [2]. The worldwide dissemination and dramatically increasing prevalence of the CTX-M family of ESBLs is due to the selective pressure caused by the over-use, and more importantly, mis-use of the β-lactam antibiotics [2].
Risk factors for an ESBL-producing UTI are; recent hospitalisation, treatment with 3rd generation Cephalosporins, old age (over 65), diabetes, recurrent UTI, indwelling catheters and female gender [3]. It has been reported that ESBL-producing isolates are more likely to be associated with significant pyuria, suggesting that ESBLs are more likely to cause a clinically significant UTI [4,5]. Although most ESBL-producing UTIs are acquired in the community, a hospitalised patient is approximately 4 times more likely to be diagnosed with an ESBL-producing UTI [3].

The significant increase in movement of livestock and agricultural produce and human travel has facilitated the rapid amalgamation and dissemination of antibiotic resistance genes [6]. The emergence and spread of antibiotic resistance determinants is often through horizontal transmission of mobile genetic elements such as plasmids. [7]

It is the large, low-copy, self-transmitting resistance conjugative plasmids that are increasingly threatening the efficacy of antibiotics for Gram-negative infections and have been directly linked to antibiotic resistance outbreaks. Conjugative plasmids can carry more than one gene for a selectable advantage such as antibiotic resistance genes and virulence factors such as bacteriocins and cytotoxins. Under antibiotic and infectious pressure, these traits may facilitate the successful spread of certain plasmid types between different bacterial hosts and geographical locations [8]. This results in ESBL-producing isolates that can show resistance to other types of antibiotics, leading to multidrug resistance (MDR). MDR limits treatment options, resulting in less possible future evolution [22]. Although most ESBL-producing UTIs are acquired in the community, a hospitalised patient is approximately 4 times more likely to be diagnosed with an ESBL-producing UTI [3].

Materials and Methods

Isolate collection and phenotypic detection

Bacterial isolates (n=236) of Enterobacteriaceae isolated from urinary tract infections were obtained from the Leicester Royal Infirmary hospital (Leicester, England). The Leicester Royal Infirmary was chosen as a collection site as a large number of samples are received from all over the Midlands and the Midlands has a large population of a wide variety of ethnic groups. According to the 2011 Census, 20.8% of people in the West Midlands and 14.6% of people in the East Midlands identify as an ethnicity other than white British [26]. Specifically in the city of Leicester, 49.5% of people identify as non-white British, of this 37.1% identify as Asian/Asian British [27]. ESBL production was confirmed phenotypically via the MAST ESBL ID double disc synergy method, conforming to British Society of Antimicrobial Chemotherapy (BSAC) standards [28]. Disks contained Cefotaxime, Ceftazidime and Cefpodoxime with an ESBL inhibitor (Clavulanic acid) counterpart. Plates were incubated for 18 hours at 37°C. A zone of inhibition difference of 5 mm or more between the β-lactam and the ESBL inhibitor clavulanic acid indicated a positive result for ESBL production. Four control isolates were obtained from Public Health England: NCTC 13353 (CTX-M-15), NCTC 13351 (TEM-3), NCTC 13368 (SHV-18) and NCTC 13442 (OXA-48) for use as controls in phenotypic and genotypic tests.

Ethical approval

Ethical approval for this study was not required as Informed consent from patients was not required, as we collected only waste material (bacterial cultures), and therefore there is no link to patient data.

DNA extraction and genotypic detection by multiplex PCR

We have previously outlined the method used for DNA extraction [29]. Genotypic identification was by means of a multiplex PCR method for the detection of the ESBL-producing genes (CTX-M, SHV, TEM and OXA). These were then further characterised into the CTX-M family (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) by adapting a multiplex PCR assay by Al-Mahayie [30]. The GoTaq G2 Flexi DNA Polymerase kit was chosen for all PCR assays (Promega, Southampton, UK). PCR amplification reactions were performed using the PikoReal® 96 well RT-PCR platform (ThermoFisher, Loughborough, UK) in a volume of 25 μl containing 5 μl buffer, 2 μl MgCl2, 0.5 μl DNTPs, 0.125 μl Taq polymerase, 400 nM of each primer, 2.5 μl of DNA template and made up to 25 μl with water.

Cycling parameters for the first multiplex assay were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min (gradual temperature decrements of 0.5°C per cycle, final annealing temperature 48°C), and 72°C for 1 min; and with a final extension at 72°C for 10 min.

Cycling parameter for the second multiplex assay were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 25 s; 52°C...
for 40s and 72°C for 50 s; and a final extension at 72°C for 6 min. Primers can be found in Table 1.

### Table 1: Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>CGG ATG GCA TGA CAG TAA GAG</td>
<td>AGG ACC ACT TCT GCG CTC G</td>
<td>[29]</td>
</tr>
<tr>
<td>SHV-18</td>
<td>CTCAAGGGATGTATGGTTGATGC</td>
<td>CTA CGA GCC GGA TAA CGC G</td>
<td>[29]</td>
</tr>
<tr>
<td>CTX-M</td>
<td>GCTCATCTATGTCGCGGACC</td>
<td>GCATCTCACTGGAGTGGACG</td>
<td>[29]</td>
</tr>
<tr>
<td>OXA-48</td>
<td>GCGAATGCCGCTGCGCTGCGAAAG</td>
<td>CAGGCCCCACACCCGAGATG</td>
<td>[29]</td>
</tr>
<tr>
<td>CTX-M-1</td>
<td>AAG AAT CAC TGC GCG GTT TC</td>
<td>AGC TTA TTC ATC GCC AGT TC</td>
<td>[30]</td>
</tr>
<tr>
<td>CTX-M-2</td>
<td>AGC TTA TCT CTC GCC AGG TG</td>
<td>CCG CCG GCA GAT TTT TCA GG</td>
<td>[30]</td>
</tr>
<tr>
<td>CTX-M-8</td>
<td>TCG CTT AAG GCC GAT GTT GC</td>
<td>CAA AGA GAG TGC AAC GGA TG</td>
<td>[30]</td>
</tr>
<tr>
<td>CTX-M-9</td>
<td>CAA AGA GAG TGC AAC GGA TG</td>
<td>ATT GGA AAG CGT TCA TCA CC</td>
<td>[30]</td>
</tr>
<tr>
<td>CTX-M-25</td>
<td>GCA CGA TGA CAT TCG GG</td>
<td>AAC CCA CGA TGT GGG TAG C</td>
<td>[30]</td>
</tr>
</tbody>
</table>

Table 1: Primers used in this study. Primers for the first multiplex PCR assay were TEM, SHV, CTX-M and OXA. The primers for the second multiplex PCR assay were CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Primers were designed using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA). Primers were then checked for specificity by the BLAST software (National Center for Biotechnology Information, Bethesda, USA). All PCR products were ran on a 2% agarose gel (UltraPure ™ Agarose, ThermoFisher Scientific, Paisley, UK) for 90 mins at 60 Volts. A GeneRuler 50 bp DNA Ladder, ready-to-use (ThermoFisher Scientific, Paisley, UK) was used for comparison.

### Multiplex PCR-based replicon typing

Each isolate was sub-cultured in Luria Bni broth overnight at 37°C. Plasmids were extracted using the Illustra PlasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Detection of plasmids was by means of adapting the replicon typing assay designed by [31]. PCR amplifications were performed in a volume of 25µl containing 5 µl buffer, 2 µl magnesium chloride, 0.5 µl DNTPs, 0.125 µl Taq polymerase, 400 nM of each primer and 2.5 µl of plasmid DNA. Cycling parameters were as follows: initial denaturation at 94°C for 5 min 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min. Primers can be found below in Table 2.

### Statistical analysis

The Chi Square test was used for statistical comparison and the Spearman Rank Correlation test was used to analyse the relationship between plasmids and ESBLs. P values < 0.05 were regarded as significant.

### Results

236 Enterobacteriaceae were tested by multiplex PCR for the ESBL genes CTX-M, TEM, SHV and OXA; and the CTX-M sub-groups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Multiplex PCR-based replicon typing tested for IncFII, IncFIA, IncL/M, IncN and IncI1.

The PCR assay assigned the four controls to their correct phylogenetic groups. ESBL genes were identified as follows: CTX-M=169 (71.6%) OXA=18 (7.6%), TEM=9 (3.8%) and SHV=9 (3.8%). Multiple resistance genes were detected in 16% of isolates, specifically...
CTX-M + OXA = 16 (6.8%), CTX-M + TEM = 12 (5.1%), CTX-M + OXA + SHV = 5 (2.1%) and CTX-M + SHV = 5 (2.1%). This can be seen in Table 1. 31 isolates did not contain any of the four genes tested.

CTX-M genes were identified as follows: CTX-M-1 = 148 (84.1%), CTX-M-9 = 22 (12.5%), CTX-M-25 = 3 (1.7%), CTX-M-8 = 2 (1.1%) and CTX-M-2 = 1 (0.6%). This can be seen in Table 2. All isolates identified as containing CTX-M in the previous assay, contained at least one of the CTX-M sub-groups tested. One isolate contained both CTX-M-1 and CTX-M-8.

This can be seen in Figure 1. 235 of the 236 isolates contained at least one plasmid (Table 3).

Multiple plasmids were found in 37% of isolates. IncL/M, IncN, IncI1, IncFII and IncFIA genes by multiplex PCR analysis. All genes were detected, however CTX-M was the most frequent.

Table 3: Frequency of ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of CTX-M, TEM, OXA and SHV genes by multiplex PCR analysis. All genes were detected, however CTX-M was the most frequent.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Number of isolates (n=236)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M</td>
<td>169</td>
</tr>
<tr>
<td>TEM</td>
<td>9</td>
</tr>
<tr>
<td>SHV</td>
<td>18</td>
</tr>
<tr>
<td>OXA</td>
<td>9</td>
</tr>
<tr>
<td>CTX-M + OXA</td>
<td>16</td>
</tr>
<tr>
<td>CTX-M + OXA + SHV</td>
<td>5</td>
</tr>
<tr>
<td>CTX-M + SHV</td>
<td>5</td>
</tr>
<tr>
<td>CTX-M + TEM</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 4: Frequency of CTX-M sub-groups in UTI isolates in the Leicestershire area. 169 isolates previously identified as containing the CTX-M gene, were tested for the presence of CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 genes by multiplex PCR analysis. All genes were detected, however CTX-M-1 was the most frequent. A combination of CTX-M-1 and CTX-M-8 was found.

A spearman rank was carried out to access the correlation between ESBL genes and plasmid type. Results can be found in Table 5. A significant positive correlation between the CTX-M gene and IncL/M, IncFII and FIA plasmids was found. Also found was a significant positive correlation between the CTX-M gene and IncL/M, IncFII and FIA plasmids was found.
positive correlation between the IncL/M plasmid and the CTX-M-8 gene. None of the other CTX-M sub-types (CTX-M-1, CTX-M-2, CTX-M-9 and CTX-M-25) had a significant correlation with any of the plasmids. No significant correlation was found for the TEM, SHV and OXA genes and any of the plasmids.

<table>
<thead>
<tr>
<th></th>
<th>CTX-M</th>
<th>TEM</th>
<th>OXA</th>
<th>SHV</th>
<th>CTX-M-1</th>
<th>CTX-M-2</th>
<th>CTX-M-8</th>
<th>CTX-M-9</th>
<th>CTX-M-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>IncL/M</td>
<td>0.200</td>
<td>-0.033</td>
<td>0.094</td>
<td>0.010</td>
<td>0.028</td>
<td>-0.042</td>
<td>0.042</td>
<td>0.013</td>
<td>-0.074</td>
</tr>
<tr>
<td></td>
<td>(0.002)</td>
<td>(0.619)</td>
<td>(0.1489)</td>
<td>(0.873)</td>
<td>(0.674)</td>
<td>(0.519)</td>
<td>(0.525)</td>
<td>(0.846)</td>
<td>(0.262)</td>
</tr>
<tr>
<td>IncN</td>
<td>0.148</td>
<td>0.026</td>
<td>0.097</td>
<td>0.007</td>
<td>0.080</td>
<td>-0.027</td>
<td>0.093</td>
<td>-0.081</td>
<td>-0.047</td>
</tr>
<tr>
<td></td>
<td>(0.023)</td>
<td>(0.694)</td>
<td>(0.1373)</td>
<td>(0.918)</td>
<td>(0.220)</td>
<td>(0.681)</td>
<td>(0.154)</td>
<td>(0.214)</td>
<td>(0.474)</td>
</tr>
<tr>
<td>IncI1</td>
<td>0.029</td>
<td>-0.067</td>
<td>-0.036</td>
<td>0.105</td>
<td>0.034</td>
<td>-0.016</td>
<td>-0.023</td>
<td>0.079</td>
<td>-0.028</td>
</tr>
<tr>
<td></td>
<td>(0.660)</td>
<td>(0.303)</td>
<td>(0.5781)</td>
<td>(0.109)</td>
<td>(0.604)</td>
<td>(0.809)</td>
<td>(0.732)</td>
<td>(0.231)</td>
<td>(0.674)</td>
</tr>
<tr>
<td>IncFII</td>
<td>0.168</td>
<td>-0.030</td>
<td>0.042</td>
<td>-0.028</td>
<td>0.016</td>
<td>-0.041</td>
<td>0.147</td>
<td>-0.037</td>
<td>-0.072</td>
</tr>
<tr>
<td></td>
<td>(0.010)</td>
<td>(0.646)</td>
<td>(0.5224)</td>
<td>(0.664)</td>
<td>(0.811)</td>
<td>(0.528)</td>
<td>(0.025)</td>
<td>(0.569)</td>
<td>(0.271)</td>
</tr>
<tr>
<td>IncFIA</td>
<td>0.170</td>
<td>-0.040</td>
<td>-0.004</td>
<td>0.127</td>
<td>0.055</td>
<td>-0.037</td>
<td>0.057</td>
<td>-0.059</td>
<td>-0.064</td>
</tr>
<tr>
<td></td>
<td>(0.009)</td>
<td>(0.543)</td>
<td>(0.9568)</td>
<td>(0.051)</td>
<td>(0.401)</td>
<td>(0.576)</td>
<td>(0.388)</td>
<td>(0.369)</td>
<td>(0.339)</td>
</tr>
</tbody>
</table>

**Table 5**: Correlation coefficients and associated level of significance for the establishing the association between the plasmids and the ESBL genes CTX-M, TEM, OXA, SHV, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Correlation was determined using Spearman Rank analysis. A value 1=perfect correlation; 0–1=no correlation; -1–0=one variable increases as the other decreases; -1= perfect inverse correlation. P values are given in brackets and P values <0.05 were regarded as significant.

**Discussion**

The major cause of antibiotic resistance in Enterobacteriaceae is ESBL-production. ESBL-producing UTIs are a major problem in the UK, due to their increasing prevalence and limited treatment options [32]. Given how common ESBL-producing infections are worldwide, prevalence studies are important to ascertain where resistance is a major problem.

In this paper, the prevalence of ESBL genes was investigated in UTIs in the Leicestershire area of the UK. In short, the CTX-M gene was found in 71.6% of samples, in comparison genes TEM, SHV and OXA were detected in ≤ 16% of samples. The most prevalent CTX-M was CTX-M-1 at 84.1%. The most common plasmid found was IncI/M at 29.2%, with IncFII following closely at 27.5%. Multiple plasmids were found in 37% of isolates, with the IncL/M, IncI1 and IncFIA combination the most common. A significant correlation between CTX-M and all plasmids except IncI1 and more specifically, CTX-M-8 and IncFIA was found, however no other gene family significantly correlated with any of the plasmids.

Here, the relevance of our findings to other studies will be evaluated within the context of the One Health Approach. The One Health Approach describes a holistic and multi-sectoral approach to antimicrobial resistance, as resistant organisms exist in humans, animals, food and the environment [33]. The main aim of the One Health Approach is to ensure that antimicrobial agents continue to be effectively by developing policies that promote the responsible use of antimicrobial agents. The development of policy decisions depend on economic and scientific evidence. Prevalence studies are an important part of this [34].

As far as we are aware, this is the first study to investigate the prevalence of ESBL-producing Enterobacteriaceae and their relationship to plasmids, in the Leicestershire area of the UK.

In this study, we found that CTX-M was the most common ESBL and the most common sub-group was CTX-M-1. This is comparable with other European countries, and is consistent with the worldwide dissemination of the CTX-M ESBL [9,15,35,36]. CTX-M-1 has been found to be the most prevalent gene in humans, animals and food in the UK, frequently found in poultry and cattle isolates [37]. It has been established that the prevalence of ESBLs differs significantly between countries. Within Europe, a high prevalence has been seen in Southern Europe, while a generally lower prevalence is seen in Northern Europe. Outside of Europe, Turkey and India have reported high levels of ESBLs [13].

The high number of CTX-M isolates found in this area may be due to the multicultural nature of the Leicestershire community. A study in a hospital in Birmingham, UK also found a high level of ESBL and suggested that this could be due to a high immigrant population [5]. One possible cause of elevated levels of ESBLs in high immigrant populations could be faecal colonisation. Transmission of faeces to the urinary tract is frequently a cause of UTI. As E.coli is a common inhabitant of the gastrointestinal tract, it is possible that CTX-M-harbouring E.coli could be a commensal bacterium that can become pathogenic upon colonization of the urinary tract [38]. To further this, a direct link between conjugative resistance plasmids in the micro flora and increased treatment failure has been found [39]. In another study in Birmingham, they found a direct link between place of birth and CTX-M-1 colonisation in stool samples. Those that were originally born in Afghanistan saw the highest colonisation at 60%, followed by those born on the Indian subcontinent (India, Pakistan, Bangladesh or Sri Lanka) with a 25% colonisation. Those that travel to certain areas were also associated with CTX-M colonisation. Travellers to South Asia (India, Pakistan, Bangladesh, Sri Lanka or Nepal) in the last year had a 38.5% colonisation, suggesting a geographical, rather than ethnic susceptibility [36].

In India, a prevalence rate of 53% for CTX-M was seen in a study detecting ESBLs in UTIs. Antibiotic usage in India is far higher than in...
the UK, due to issues with lack of restriction in antibiotic prescribing which could explain why they saw a higher incidence of CTX-M [40].

Prevalence rates of CTX-M have been seen as high as 94.4% in Iran, 73% in Mexico, 98.7% in Japan and 100% in Ghana [36]. These studies show that the problem of ESBL producing infections is not limited to the UK. As bacteria do not recognise geographical borders, so too does our approach to tackling AMR need to be borderless.

We found that 16% of isolates contained more than one ESBL type. Interestingly, in all of the multi-ESBL isolates CTX-M was always present, this suggests that CTX-M may play a key role in the production of MDR bacteria. Of these, CTX-M and OXA were the most common multi-ESBL, detected in 16 isolates. 2% of isolates contained three genes, specifically, CTX-M, OXA and TEM. It has been reported that CTX-M-15 combined with an OXA-30 gene can survive β-lactamase inhibitors, since OXA-30 is poorly inhibited and also confers resistance to cefepime [12]. Although we did not specifically type the ESBL genes in these isolates, it is possible that it could be this combination present. The coexistence of CTX-M and other antibiotic resistance genes could be one of the reasons why CTX-M has been so successful. This has been found elsewhere, with incidences of combinations of ESBLs as high as 95.4%, with regards to the CTX-M and SHV combination [41,42]. Our combination of three genes has also been found elsewhere [42]. Furthermore, other Multi ESBL combinations have also been found in animals and the environment, suggesting that multiple ESBL combinations are not limited to humans. This data combined shows that multi ESBLs are widespread and could pose a significant threat [4,43].

A similar incidence of ESBLs has been reported in livestock and companion animals globally [44-47]. In a study looking at canine UPEC in Switzerland, CTX-M-1 was found in 28.6% of ESBL-producing dogs, suggesting that dogs, a common companion animal worldwide, may be a reservoir for CTX-M-1 spread into humans [47]. With raw meat a specialty in some countries and the rise of raw food diets in both humans and companion animals, a high level of ESBLs in livestock is a cause for concern. Both livestock and companion animals could be considered a major vector of ESBL-producing bacteria [37]. As it has been suggested that the majority of ESBL infections are community-acquired, this could suggest a relationship between companion animals, livestock and humans.

This study focused on the prevalence of IncI1/M, IncFII, IncI1, IncFIA and IncN. These conjugative plasmids have previously been associated with the dissemination of antibiotic resistance determinants such as ESBLs, in Enterobacteriaceae and other bacteria.

Whilst IncI1/M was the most common plasmid found in the isolates tested, when IncFII and IncFIA (both members of the IncF group) are combined, the frequency is far higher at 50.8%. The IncF group is one of the most common plasmids found in UTI isolates and is frequently associated with CTX-M [12]. Studies show that IncI1/M is also highly common, and both have been termed as “epidemic” plasmids [11]. Both of these plasmids can persist for months without the need for selective pressure by antibiotic usage [39]. This suggests that simply reducing antibiotics by antibiotic stewardship alone will not reduce the prevalence of these plasmids.

The distribution of plasmids harbouring ESBL genes in this population differs from the distribution described in other studies. Studies have agreed that CTX-M is associated with IncN, IncFII and IncI1/M, however reports have shown that CTX-M is also associated with IncI1. IncI1 plasmids harbouring CTX-M have been described in the UK, US, Belgium, Netherlands, France and Australia in isolates from horse, cattle and human isolates [16]. Studies have also found that other ESBL types are associated with particular plasmids. It has been found that SHV can be associated with IncFII, IncI1/M and IncI1. Likewise, TEM can be associated with the same plasmids, and additionally IncI1 [19]. A reason for the differing results could be due to different antibiotic and environmental selection pressures in this population, leading to selection of CTX-M harbouring IncI1/M, IncF, and IncN plasmids, but not IncI1 plasmids.

In this study, no significant correlation between CTX-M-1 (the most common CTX-M subtype) and any other plasmid was found. There are conflicting reports within the literature with regards to the most significant association between CTX-M-1 and plasmid types. Some reports suggest a correlation between CTX-M-1 and IncFII, whilst others, mainly in Europe, suggest a correlation between CTX-M-1 and IncI1. Other reports suggest no overall correlation at all [37,48]. It appears that the association between CTX-M-1 and plasmids differs between geographical locations and sample type. These differences in reports could be attributed to socioeconomic factors, animals and number of samples. It could be suggested that CTX-M-1 is equally adapted to most plasmid types therefore a significant correlation between just one plasmid was not seen. It could also be suggested that the high level of CTX-M-1 in this population is also due to another mechanism other than conjugative plasmids, such as chromosomally mediated resistance.

A significant correlation between CTX-M-8 and IncFII was found. Whilst CTX-M-8 is not the highest prevailing gene found in this study, it is a significant finding, as IncFII was very common. A link has been found between CTX-M-8 harbouring IncI1 plasmids and companion animals. This could further strengthen the argument that CTX-M genes could be passed, via plasmids, from companion animals to humans [46].

Though studies suggest that IncN does not contribute to the prevalence of CTX-M in humans to the same extent as incI1, this was not the case in this study, as IncN was seen to be more prevalent than IncI1 and a significant correlation was found [21]. This theory has also been contradicted by another study that found that IncN was in fact associated with humans and 95.5% of isolates containing IncN harbouring CTX-M-1 [37]. In a Danish study, IncN plasmids carrying CTX-M-1 were seen in both pigs and farm workers, and it was demonstrated that these plasmids were transmitted within the farm, showing animal to human transmission of these plasmids harbouring CTX-M-1 [11].

Multi-replicon plasmids were found in 37% of isolates. Multiple plasmids frequently occur in the same bacterial cell, however, cross-interference between plasmid replicons guarantees that the most closely related plasmids are incompatible and cannot stably persist together [39]. When multi-replicon plasmids occur, generally one replicon is usually highly conserved, and the other is free to diverge. This gives the bacterial host the benefit of being able to change antibiotic resistance determinants and virulence factors, depending on antibiotic pressure and environment [16]. Multi-replicons have been found in other UTI isolates, and multi-replicons containing multiple IncF types appear to be common [12]. Multi replicons are also common in the environment. A study found that the diversity of plasmids found in the environment was higher than in UTIs in the same area. CTX-M-Is in the environment have been shown to be associated with IncI1 and IncN [43]. A report has suggested a direct link between wastewater treatment plants and ESBL-producing UTIs.
Sewage sludge used for agriculture may be one of the ways that ESBL-producing isolates enter the food chain [43]. It has been established that CTX-M-1 harbouring E.coli in manure spread on fields can survive in the soil for at least a year. This demonstrates the capability of antibiotic resistant bacteria to survive under environmental conditions in the absence of antibiotic selection pressure [21].

A limitation of this study is that isolates were not categorised into source of isolate (community/hospital acquired). However, other studies suggest that the CTX-M family and ESBLs in general are associated with community-acquired infection [4,49]. In this study, we only collected ESBL-positive isolates. From this, we cannot determine the level of ESBL resistance as a whole. This study should be followed up regularly, to study the trends of antibiotic resistance in this area. More surveillance studies on animal and environmental isolates are needed, to fully understand the relationship between these and human infections.

We have reported similar results to what has been found to be present in the rest of the UK and worldwide. CTX-M-harbouring Enterobacteriaceae is a major threat to patient care and healthcare costs. Genotypic prevalence studies are an important contribution to the understanding and prediction of antibiotic resistance evolution and antibiotic stewardship. Knowledge of plasmid types circulating in bacterial populations is vital to advancing a new prospective to control these plasmids, such as replicon-targeting compounds. A strategy to prevent the further dissemination of these plasmids needs to be implemented.

Combined, these findings highlight the importance of restricting antibiotics sales to only those with medical prescriptions and appropriate use, as many low and middle-income countries have not yet enforced policies that prevent widespread self-medication with antibiotics.

This study reinforces the One Health approach and underpins the importance of antibiotic stewardship and infection prevention schemes in humans, animals, food production and the environment in order to limit the spread of ESBL-harbouring Enterobacteriaceae, not only in the UK, but worldwide.

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Conflict of Interest

The authors declare no conflict of interest.

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References
