Molecular epidemiology of extraintestinal pathogenic *Escherichia coli* isolates from a regional cohort of elderly patients highlights the prevalence of ST131 strains with increased antimicrobial resistance in both community and hospital care settings

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Objectives: To assess the molecular epidemiology and prevalence of antibiotic resistance in *Escherichia coli* causing urinary tract infections of elderly patients from community and hospital settings. Also, to determine whether the possession of antibiotic resistance and virulence-associated genes can be linked to patient location or the clonal group of the organisms in question.

Methods: *E. coli* were isolated from the urine samples of elderly patients from the Nottingham area, and subjected to antibiotic susceptibility testing, virulence gene detection by PCR and multilocus sequence typing.

Results: No correlation was observed between community- or hospital-derived strains with regard to antibiotic resistance levels or virulence gene profiles. *E. coli* ST131 (where ST stands for sequence type) was the predominant ST found in both hospital and community samples, and demonstrated high levels of antibiotic resistance to the test panel, but did not possess a significantly larger array of virulence genes or a specific gene profile compared with other STs.

Conclusions: The level of antibiotic resistance or virulence gene possession in uropathogenic *E. coli* is not directly associated with the healthcare setting of the patient, but there is a variation in antibiotic resistance and virulence gene possession depending on clonal group. ST131 is highly virulent and demonstrates high levels of antibiotic resistance, but its virulence does not appear to be attributable to the possession of a specific virulence-associated gene set or the possession of any virulence-associated gene in significantly higher levels than in any other ST.

Keywords: MLST, ExPEC, UTIs

Introduction

Urinary tract infection (UTI) is one of the most common nosocomial infections in the UK and also has a significant occurrence in the community.¹,² It is well known that *Escherichia coli* is the primary aetiological agent of UTI in all age ranges, accounting for up to 80% of infections.³,⁴ Although once considered to be a clonal organism, it is now widely accepted that *E. coli* as a species is extremely heterogeneous and is subject to high rates of recombination within the accessory genome.⁵ Due to this high level of heterogeneity in the species, *E. coli* are broadly categorized into three main groups: (i) commensal *E. coli*; (ii) intestinal *E. coli*, such as enterotoxigenic *E. coli*, enteropathogenic *E. coli* and enterohaemorrhagic *E. coli*; and (iii) extraintestinal pathogenic *E. coli* (ExPEC). From this level of classification, *E. coli* are usually further subdivided into pathotypes on the basis of the isolation site and the possession of certain ‘virulence factors’. For example, within ExPEC, *E. coli* causing UTI are termed uropathogenic *E. coli* (UPEC) on the basis that they were isolated from the bladder and possess at least two UPEC-associated virulence factors.⁶ Although it is now appreciated that the level of heterogeneity within this species is immense, in a clinical setting UPEC are still traditionally classed and treated as a homogenous group of organisms. Due to the era of multilocus sequence typing (MLST), we now know that *E. coli* strains within a population can vary greatly in terms of their...
evolutionary descent, which in turn can affect their pathogenic potential and fitness in an infection. This is especially so in the case of the recently emerged ST131 (where ST stands for sequence type), which has been linked to community acquisition and, more significantly, the worldwide dissemination of CTX-M-15. It has been well reported that a reservoir of extended-spectrum β-lactamase (ESBL)-producing organisms that usually cause UTI exists in long-term care facilities, such as nursing homes. This will inevitably pose a greater risk to elderly patients living within long-term care facilities. Previous findings by our group reported that significant variations exist within a population of E. coli causing UTI in the Nottingham area, and that a subgroup of organisms with increased virulence potential and antimicrobial resistance existed within this population. This investigation aimed to compare the molecular epidemiology, antimicrobial activity and virulence-associated gene (VAG) carriage of the aforementioned E. coli isolated from two populations within the same geographical area, namely the community setting and the hospital setting. The study also aimed to determine whether any observed differences could be attributed to the clonal group or the possession of a specific VAG set.

Methods

Bacterial isolates

Two hundred and fifty urine culture plates were collected at random from Nottingham University Hospitals (NUH) between October 2008 and June 2009. Cultures were collected anonymously (therefore no ethical approval or informed consent was required) from both hospitalized and community patient samples sent to the NUH clinical laboratory from inpatient and outpatient departments. No supportive clinical information regarding symptoms was available to confirm the diagnosis of UTI rather than colonization. All bacterial species were identified using API identification kits (bioMérieux).

MLST

MLST was carried out on 121 of the 150 E. coli isolates from urine cultures using the Achtman typing scheme (http://mlst.ucc.ie/mlst/dbs/EColi), adhering to the protocols published on the web site. Briefly, the seven house-keeping genes adk, fumC, gyrB, icd, mdh, recA and purA were amplified using a PCR protocol, and the amplicons sequenced using the amplification primers. Individual gene sequences were searched against the MLST database and assigned an allelic profile number. ST designations were based upon the combination of the seven alleles that each strain possessed. BioNumerics v.6.5 was used to generate a minimum spanning tree from non-concatenated sequences of the seven alleles. Therefore, the minimum spanning tree that was generated illustrates clusters of closely related organisms in terms of the gene allele types present. Strains that share the same alleles theoretically evolved from a recent common ancestor, but this method does not weight single base changes within each gene and therefore does not attempt to infer a phylogenetic relationship between each strain.

Antibiotic susceptibility testing

Antibiotic susceptibility profiles were obtained for all isolates using breakpoint antibiotic media. The BSAC method for antimicrobial susceptibility testing was followed to prepare standardized inocula. The antibiotic panel used was as follows: gentamicin (2 mg/L); cefotaxime (1 mg/L); ceftazidime (1 mg/L); meropenem (2 mg/L); piperacillin/tazobactam (16 mg/L); co-amoxiclav (32 mg/L); trimethoprim (2 mg/L); ciprofloxacin (4 mg/L); cefadroxil (32 mg/L); nitrofurantoin (32 mg/L); and amoxicillin (32 mg/L). All strains were tested for the ESBL production phenotype using ESBL combination ID discs (MAST). All isolates were also screened for the presence of the β-lactamase genes blaTEM, blaSHV, blaCTX-M and blaOXA, using a previously described multiplex PCR. Reference strains E. coli NCTC 13351, E. coli NCTC 13353 and Klebsiella pneumoniae NCTC 13368 were included as controls. For the typing of CTX-M genes, PCR products were amplified using a previously published protocol and amplicons were sequenced using the amplification primers.

Detection of VAGs

The presence of 30 VAGs was determined using previously published multiplex PCR protocols. VAGs include genes involved in iron acquisition, adhesion, toxin production and pathogenicity island markers among others.

Cell cultures

T24 human epithelial cells (HPA cultures) were grown in McCoy’s 5A modified medium (Sigma, UK) supplemented with 10% fetal bovine serum (Sigma, UK) and 0.75% l-glutamine (Sigma, UK). Cells were grown in a 5% CO₂ atmosphere at 37°C and subcultured twice weekly. Two days prior to cell infection assays, the T24 cells were seeded into 24-well plates.

Association and invasion assays

All strains were assayed for their ability to invade human bladder epithelial cells using a classical gentamicin protection assay, as described previously. Assays were performed in triplicate wells in each assay and in duplicate on different days. Bacteria were cultured overnight in LB broth, harvested by centrifugation and resuspended in supplemented tissue culture medium. The bacterial cell density was then adjusted to 2 x 10⁷ cfu/mL, giving a multiplicity of infection of 1:100. The invasive ExPEC reference strain CFT073 was used as a positive control strain in all assays and E. coli DH5α was used as a negative control strain. The mean number of invasive bacteria was determined by Miles and Misra plate counts from triplicate wells. Strains that showed a >10-fold increase in invasion compared with CFT073 were deemed to be strains of limited invasive potential.

Statistical analysis

Statistical analysis and the production of heat maps were performed using SPSS PASW statistics software (version 18.0). A combination of parametric and non-parametric tests was applied, depending on the format of the data in question. χ² tests were performed to compare bacterial prevalence in different patient groups, the distribution of antibiotic resistance between hospital and community sources, the prevalence of antibiotic resistance to individual antibiotics between ST131 and non-ST131 strains, the possession of CTX-M and OXA ESBLs between ST131 and non-ST131 strains, and the possession of VAGs among strains of varying invasive potential. The Kruskal-Wallis test for independent samples was used to determine whether the distribution of antibiotic resistance was equal across different ExPEC clonal groups. The Student’s t-test was used to determine whether invasion levels were significantly different between ST131 and non-ST131 strains.
Results

*E. coli isolated from urine samples of elderly patients are an epidemiologically diverse population*

Two hundred and fifty urine culture plates were collected from NUH, of which 158 originated from the community and 92 were from hospitalized patients. *E. coli* was present in 60% (150/250) of sample cultures compared with *Enterococcus faecalis* (45%), *Proteus mirabilis* (23%), *Pseudomonas aeruginosa* (20%) and *Staphylococcus aureus* (18%). There was a significant difference between the prevalence of *E. coli* in male (50/117) and female (100/133) cultures (*P* < 0.001), but no significant difference in the prevalence of *E. coli* in samples from hospitalized patients (55%) or the community (63%). STs were obtained for 121 *E. coli* isolates and uploaded to the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). A total of 52 STs were identified within the strain collection, including 11 novel STs. The most highly represented STs within the strain collection belonged to the ST131 complex, which includes ST131, ST1461 and the novel ST1982, and accounted for 22% of the isolates. ST73 and ST69 accounted for 11% and 9% of isolates, respectively. A predominant ExPEC ST complex present in the MLST database, the ST95 complex, was under-represented in this strain set (3% of isolates). The ST131 complex showed a strong association with the community, with 78% of isolates originating from patients within the community, compared with 22% of samples originating from hospitalized patients (Figure 1); this association, however, was not significantly different from observed distributions in other STs.

![Minimum spanning tree of E. coli STs from elderly patients with UTI. Grey shading indicates proportion of isolates within STs derived from hospital environments and unshaded areas indicate the proportion of isolates within STs derived from community environments. Strains within the same ST possess seven identical alleles, whereas a single locus variant possesses one different allele to the other ST and a double locus variant differs by two alleles. Novel STs from this study are shown in bold.](image-url)
E. coli from hospital and community environments do not exhibit significantly different levels of antibiotic resistance or VAG carriage, but do show an association with CTX-M carriage

The highest levels of resistance were observed against ampicillin (45% of strains), trimethoprim (41%), cefradine (26%) and ciprofloxacin (21%). Slight differences were observed between the resistance levels of hospital and community isolates, although these differences were not statistically significant. With regard to β-lactamase gene carriage, 6% of the E. coli isolates harboured blaSHV, 52% harboured blaTEM (although it is not known what proportion of these fall into the extended-spectrum category), 11% harboured blaCTX-M and 7% harboured blaOXA, with no statistically significant difference between hospital and community samples. The most frequently detected VAGs in the strain set (Figure 2b) were the adhesin fimH (113/121), iron acquisition genes fyuA and iutA (98/121 and 64/121, respectively), the pathogenicity island marker PAI (85/121), the serum resistance gene traT (70/121), and the type II capsule marker kpsMT II (65/121). The possession of subunits of the pap operon, papA, papC and papG, which are associated with adhesion to the upper urinary tract, was markedly less than the possession of fimH, which is involved in adhesion to the lower urinary tract (30%–40% of isolates carried pap genes compared with 94% for fimH). This difference was observed across the strain set, and no significant difference was observed in the prevalence of any gene between community- and hospital-derived strains.

There is evidence of a correlation between clonal group and antibiotic resistance

The distribution of resistance to trimethoprim, ciprofloxacin, ampicillin and cefradine was not evenly distributed across different clonal groups (Kruskal–Wallis test for independent samples, P<0.05). More specifically, the largest percentage of resistant strains (classed as strains with the widest range of resistances to the 11 antibiotics on the panel) belonged to ST131 (Figure 3a). Within the ST131 complex, 74% of isolates were classed as resistant to trimethoprim and ciprofloxacin, and 70% were resistant to ampicillin. Thirty-seven percent of isolates within the ST131 complex were resistant to more than five antibiotics on the panel. When compared with non-ST131 isolates, the ST131 complex strains were significantly more resistant to cefotaxime (P=0.012), ampicillin (P=0.004), trimethoprim (P<0.001) and ciprofloxacin (P<0.001, χ² with
95% confidence). Interestingly, the ST131 complex also had a high carriage rate of β-lactamase genes, accounting for 25% of blaTEM strains, 46% of blaCTX-M strains and 63% of blaOXA strains. When compared with non-ST131 strains, the ST131 complex possessed significantly more blaCTX-M (P = 0.009) and blaOXA (P = 0.014). Overall, 26% of ST131 strains possessed blaCTX-M, which was the highest prevalence among the STs from this study. For this reason, all blaCTX-M genes present in ST131 strains were typed and all were subsequently confirmed to be CTX-M-15. Interestingly, the only strains that demonstrated phenotypic ESBL activity were the E. coli ST131 that possessed CTX-M-15. Other blaCTX-M strains from different clonal groups demonstrated no phenotypic extended-spectrum resistance.

Figure 3. (a) Heat map of antimicrobial resistance distribution across predominant E. coli STs. Darker shaded areas indicate higher prevalence. blaTEM, TEM β-lactamase gene; blaSHV, SHV β-lactamase gene; blaCTX-M, CTX-M β-lactamase gene; blaOXA, OXA β-lactamase gene; GEN, gentamicin; CTX, cefotaxime; CAZ, ceftazidime; MEM, meropenem; TZP, piperacillin/tazobactam; AMC, co-amoxiclav; TMP, trimethoprim; CIP, ciprofloxacin; RAD, cefradine; NIT, nitrofurantoin; AMP, ampicillin. (b) Heat map of VAG distribution across predominant E. coli STs.
There is no correlation between invasive potential and virulence gene carriage

Although some genes, such as fimH, iutA, fyuA, kpsMT II and traT, and the pathogenicity island were highly prevalent across all STs, the amount of VAGs possessed varied according to ST. STs 12, 1262 and 127, which exhibit low levels of antibiotic resistance, possessed the highest levels of VAGs (11, 10 and 9 VAGs on average, respectively). The highly antibiotic-resistant ST131, in comparison, possessed on average only six VAGs, and these were all VAGs common to the other STs. The ST131 complex does not appear to possess a specific gene set to which its highly virulent nature can be attributed. To determine whether the presence of a specific set of virulence factors had an impact on the invasive and therefore pathogenic nature of the bacterium, the invasive potential of the strains was determined using gentamicin protection assays. Bacterial strains were designated as highly invasive if they demonstrated levels of invasion >10-fold higher than that of the reference strain, CFT073, which exhibited variation of <1 log across all assays performed (<10-fold). Strains that demonstrated invasion levels 10-fold lower than that of CFT073 were deemed of limited invasive potential. In total, 24 strains (20%) exhibited a low invasive phenotype, 48 (40%) invaded within a 1 log range of CFT073 and 49 (40%) were designated as highly invasive. A highly invasive phenotype could not be attributed to a specific set of VAGs. However, sfa/focDE, papC and papG allele III were significantly more prevalent in strains with limited invasive potential than in those with a highly invasive phenotype (P<0.001, P=0.004 and P=0.01, respectively, χ² with 95% confidence; Figure 4).

Within the ST131 complex, 56% of strains exhibited a highly invasive phenotype, 30% demonstrated an invasive capability within a 1 log range of that of CFT073 and 16% demonstrated limited invasive potential. When compared with the rest of the population, strains within the ST131 complex did not invade to significantly higher numbers (Student’s t-test, 95% confidence, P=0.122).

Discussion

This investigation set out to characterize a population of E. coli isolated from urine samples of hospitalized and community elderly patients within the Nottingham area. It was found that the population was extremely diverse epidemiologically and consisted of 52 different STs. The most frequently encountered clonal group was the ST131 complex, which has links to the community and ESBL production. It has been well reported that a reservoir of ESBL-producing organisms, which usually cause UTI, exists in the community in long-term care facilities.7,12,18 Whereas no significant difference was observed in the antimicrobial resistance patterns of the two groups, the majority of CTX-M gene carriage was in the group from the community location. This can be linked to the high representation of ST131 within this dataset, as E. coli ST131 is known to be responsible for the worldwide dissemination of CTX-M-15.10,11,19 Within the ST131 complex, significantly higher levels of resistance to the front-line antibiotics ciprofloxacin, trimethoprim, ampicillin and ceftaxime were observed when compared with non-ST131 strains. The ST131 complex also had a significant association with CTX-M and OXA gene carriage. It is not surprising that all bla<subCTX-M</sub> genes detected in ST131 were CTX-M-15, but it is somewhat surprising, however, that a higher rate of carriage was not observed.7,20 It is reported that CTX-M-15 is only associated with certain ST131 clusters, differentiated by pulse type,21 and this is the focus of further investigation to determine whether a specific cluster is over-represented in this population.

With regard to VAG possession, it was found that fimH, iutA, fyuA, kpsMT II, traT and PAI were present in high levels across the whole population, and no individual VAG profiles could be perceived for any of the STs. The STs that possessed the most VAGs on average were those that possess low levels of antibiotic resistance (ST12, ST127 and ST1262). It has been suggested that ST131 goes against the age-old ethos that a pathogen can be either antibiotic resistant or possess high levels of virulence, due to the enormous cost of maintaining both traits,21,22 with previous studies linking ST131 with an increased level of VAG carriage compared with ExPEC of other ST complexes.23 In this study, however, the ST131 complex, which is highly antibiotic resistant, does not seem to possess any specific set of VAGs and it does not possess those VAGs common to other UPEC in significantly higher levels than the other STs. This would suggest that the key to ST131’s elevated virulence potential cannot be generalized to a specific VAG set or increased carriage of VAGs in general. The general hypothesis that ST131 strains exhibit increased virulence that cannot be attributed to a specific
gene set was tested by comparing the VAG profiles of a group of strains with high, normal and limited invasive potential. Strains within the ST131 complex did not exhibit an invasive potential at significantly higher levels than other ExPEC strains from different ST complexes. This not only suggests that the high levels of virulence reported in ST131 are not a result of invasive potential, but that they also cannot be attributed to the possession of any unique VAGs or the possession of any VAGs to a greater extent than in other ExPEC strains. It is clear that an in-depth study of the specific genomic content of the ST131 population is required and this is the focus of ongoing work in our laboratory.

This investigation found that vast levels of heterogeneity can be observed within a single population of E. coli causing UTI of the elderly. Any association between patient location and antibiotic resistance was attributed to the prominent presence of the ST131 complex within the dataset, which exhibited significantly higher levels of antibiotic resistance and ESBL gene carriage than any other ST. No individual VAG profiles specific to STs were observed, and the ST131 complex does not possess any VAG at higher levels than other STs and VAG possession or invasive potential could not be seen to be responsible for the reported high levels of virulence in the ST131 complex.

In conclusion, this study further exemplifies the increasing problem posed by E. coli ST131 to human health in terms of incidence and antimicrobial resistance, and highlights that the organism is also a problem in healthcare settings as well as in the community. This study also clearly highlights that ST131 populations cannot be considered as homogeneous pathogens, and that there is variation being reported in VAG carriage and antimicrobial resistance among ST131 populations from different geographical locations. Finally, our data suggest that ST131 strains do not possess increased VAG carriage or a particular subset of VAGs that account for their increasing incidence and potentially increased pathogenesis, and that a fully detailed examination of this group of organisms at both a genotypic and phenotypic level is a priority research area.

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Transparency declarations
None to declare.

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