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Molecular epidemiology of Extra-Intestinal Pathogenic *E. coli* isolates from a regional cohort of elderly patients highlights prevalence of ST131 strains containing increased antimicrobial resistance in both community and hospital care settings

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Abstract

Objectives

To assess the molecular epidemiology and prevalence of antibiotic resistance in *E. 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 clonal group of the organisms in question.

Methods

E. coli were isolated from the urine samples of elderly UTI from the Nottingham area and subjected to antibiotic susceptibility testing, virulence gene detection by PCR and MLST analysis.

Results

No correlation was observed between community or hospital derived strains with regards to antibiotic resistance levels or virulence gene profiles. *E. coli* ST131 was the predominant sequence type 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, nor a specific gene profile compared to other sequence types.

Conclusions

The level of antibiotic resistance or virulence gene possession in uropathogenic *E. coli* is not directly associated with the health care 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 attributed to the possession of a specific virulence associated gene set, nor the possession of any VAG in significantly higher levels than any other sequence type.

Introduction

Urinary tract infection 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 *E. coli* is the primary etiologic 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; (1) commensal *E. coli*, (2) intestinal *E. coli* such as ETEC, EPEC, EHEC and (3) Extra-intestinal Pathogenic *E. coli* or ExPEC. From this level of classification *E. coli* are usually further sub-divided into pathotypes on the basis of isolation site and the possession of certain "virulence factors". For example within ExPEC, *E. coli* causing UTI are termed UPEC or uropathogenic *E. coli* 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 we now know that *E. coli* strains within a population can vary greatly in terms of their evolutionary descent, which in turn can affect pathogenic potential and fitness in an infection. This is especially so in the case of the recently emerged ST131, which has been linked to community acquisition, and more significantly the world-wide dissemination of CTX-M-15.[7-11]

It has been well reported that a reservoir of ESBL-producing organisms that usually cause urinary tract infections exists in long-term care facilities such as nursing homes.[7,12] 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.[13] 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 and the hospital

setting and to determine if any observed differences could be attributed to clonal group or the possession of a specific virulence associated gene 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 required) from both hospitalised and community patients aged 70 and over. The term 'hospitalised patient' is used to describe samples sent to the NUH clinical laboratory from inpatient departments and the term 'community patient' is used to describe patient samples from GP's surgeries and out patient departments. All bacterial species were identified using API identification kits (Biomerieux).

Multilocus Sequence Typing

MLST was carried out on 121 *E. coli* isolates using the Achtman typing scheme (http://mlst.ucc.ie/mlst/dbs/E*coli*), adhering to the protocols published on the website. 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 database and assigned an allelic profile number. Sequence type (ST) designations were based upon the combination of 7 alleles that each strain possessed. Bionumerics v.6.5 was used to generate a minimum spanning tree (MST) from non-concatenated sequences of the 7 alleles. Therefore the MST illustrates clusters of closely related organisms and demonstrates the level of heterogeneity in terms of allele types present and not single nucleotide polymorphisms or phylogenetic relationship.

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 standardised inocula.[14] The antibiotic panel used was as follows; Gentamicin (2µg/ml), Cefotaxime (1 µg/ml), Ceftazidime (1 µg/ml), Meropenem (2 µg/ml), Piperacillin-tazobactam (16 µg/ml), Co-amoxiclav (32 µg/ml), Trimethoprim (2 µg/ml), Ciprofloxacin (4 µg/ml), Cephradine (32 µg/ml), Nitrofurantoin (32 µg/ml), and Amoxicillin (32 µg/ml). 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, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA}$ using a previously described multiplex PCR.[15] 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,[16] and amplicons were sequenced using the amplification primers.

Detection of virulence associated genes

The presence of 30 virulence associated genes (VAGs) was determined using previously published multiplex PCR protocols,[17] which include genes involved in iron acquisition, adhesion, toxins and pathogenicity island markers among others.

Cell cultures

T24 human epithelial cells (HPA cultures) were grown in McCoy's 5A modified media (Sigma, UK) supplemented with 10% fetal bovine serum (Sigma, UK) and 0.75% L-glutamine (Sigma, UK). Cells were grown in a 5% CO_2 atmosphere at 37^{o}C and sub-cultured 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.[13] 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 re-suspended in supplemented tissue culture medium. Bacterial cell density was then adjusted to 2x10^{7} cfu/ml, giving an MOI 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 & Misra plate counts from triplicate wells. Strains that showed more than a 10-fold increase in invasion compared to CFT073 were classed as highly-invasive strains. Those that showed more than a 10-fold decrease in invasion compared to CFT073 were deemed to be strains of limited invasive potential.

Statistical analysis

Statistical analysis and production of heat maps was performed using SPSS PASW statistics software (version 18.0). χ^2 tests were performed to compare bacterial prevalence in different patient groups and the distribution of antimicrobial resistance and VAGs among strain types.

Results

UTI of the elderly is caused by an epidemiologically diverse population of *E. coli*

Of the 250 urine culture plates collected from NUH, 158 originated from the community and 92 were from hospitalised patients. *E. coli* was present in 60% of sample cultures compared to *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 hospitalised patients (55%) or the community (63%). Sequence types (STs) were obtained for 121 *E. coli* isolates and uploaded to the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/E*coli*). A total of 52 STs were identified within the strain collection, including 11 novel STs. The most highly represented STs within the strain collection were 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 sequence type complex present in the MLST database, the ST95 complex was underrepresented 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 from hospitalized patients (figure 1); this association however was not significantly different to observed distributions in other sequence types.

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%), cephradine (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 regards to β-lactamase gene carriage 6% of the *E. coli* isolates harboured bla_{SHV}, 52% bla_{TEM} (although it is not known which proportion of these fall into the extended spectrum category), 11% possessed $bla_{CTX\text{-}M}$, and 7% bla_{OXA}. Community strains possessed CTX-M significantly more frequently than their counterparts of hospital origin (P=0.008). 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), the pathogenicity island marker PAI (85/121), the serum resistance gene *traT* (70/121), and the type II capsule marker *kpsMT*II (65/121). Possession of sub units of the *pap* operon, *papA, papC* and *papG*, which are associated with adhesion to the upper urinary tract, was markedly lower than *fimH* possession, which is involved in adhesion to the lower urinary tract (30-40% of isolates carried *pap* genes compared to 94% *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 cephradine 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 over five antibiotics on the panel. When compared to non-ST131 isolates, the ST131 complex strains are significantly more resistant to cefotaxime (P=0.012), ampicillin (P=0.004), trimethoprim (P<0.001) and ciprofloxacin (P<0.001). Interestingly the ST131 complex also had a high carriage rate of β-lactamase genes, accounting for 25% of bla_{TEM}^{+} strains, 46% of $bla_{CTX\text{-}M}^{+}$ strains and 63% of bla_{OXA}^{+} strains. When compared to non-ST131 strains, the ST131 complex possesses significantly more $bla_{CTX\text{-}M}$ (P=0.009) and bla_{OXA} (P=0.014). Overall 26% of ST131 strains possessed $bla_{CTX\text{-}M}$, which was the highest prevalence of any of the sequence types from this study. For this reason all $bla_{CTX\text{-}M}$ genes present in ST131 strains were typed and were all 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 $bla_{CTX\text{-}M}$+ strains from different clonal groups demonstrated no phenotypic extended spectrum resistance.

There is no correlation between invasive potential and virulence gene carriage

Although some genes such as *fimH, iutA, fyuA, kpsMTII II, traT* and the pathogenicity island were highly prevalent across all sequence types, the amount of VAGs possessed varied by sequence type. ST's 12, 1262 and 127, which exhibit low levels of antibiotic resistance, possess the highest levels of VAGs (11, 10 and 9 VAGs on average respectively). The highly antibiotic resistant ST131 in comparison possessed on average only 6 VAGs, and these were all VAGs common to the other sequence types. 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 over 10-fold higher than that of the reference strain, CFT073, which exhibited variation of less than 1 log across all assays performed (less than 10 fold). Strains that demonstrated invasion levels 10-fold lower than CFT073 were deemed those 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, s*fa/focDE, papC* and *papG* allele III were significantly more prevalent in strains with limited invasive potential than those with a highly invasive phenotype (P<0.001, P=0.004 and P=0.01 respectively, X^2 with 95% confidence).

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 14% demonstrated limited invasive potential. When compared to 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 characterise a population of *E. coli* causing UTI in hospitalised and community elderly patients within the Nottingham area. It was found that the population was extremely diverse epidemiologically and consisted of 52 different sequence types. 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 urinary tract infections, 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, there was an association with CTX-M gene carriage and the community location. This can be linked to the high representation of ST131 within the dataset as *E. coli* ST131 is known to be responsible for the world-wide 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 cefotaxime was observed when compared to non-ST131 strains. The ST131 complex also had a significant association with CTX-M and OXA gene carriage, thus explaining the significant association with CTX-M and the community. It is not surprising that all $bla_{\text{CTX-M}}$ 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 if a specific cluster is over-represented in this population.

With regards to virulence associated gene possession it was found that *fimH, iutA, fyuA, kpsMTII, traT* and PAI were present in high levels across the whole population, and no individual VAG profiles could be perceived for any of the sequence types. The sequence types 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,[22,23] with previous studies linking ST131 with increased level of VAG carriage compared to ExPEC of other ST complexes.[24] In this study however the ST131 complex, which is highly antibiotic resistant, does not seem to possess any specific set of VAGs nor does it possess those VAGs common to other UPECs in significantly higher levels than the other sequence types. This would suggest that the key to ST131's elevated virulence potential cannot be generalised to a specific VAG set or increased carriage of VAGs in general. The general hypothesis that ST131 strains exhibit increased virulence which 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 also cannot be attributed to the possession of any unique VAGs or the possession of any VAGs to any greater extent than that of other ExPEC strains. It is clear that an in depth study of the specific genomic content of the ST131 population is required, and 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 sequence type. No individual virulence associated gene profiles specific to sequence types were observed and the ST131 complex does not possess any VAG at higher levels than other sequence types, nor could VAG possession or invasive

potential be seen to be responsible for the reported high levels of virulence in the ST131 complex. In short *E. coli* ST131 continues to emerge as an increasing threat to human health due to its increasing prevalence and high rates of antimicrobial resistance, but there is no clear pattern of virulence associated gene carriage which marks the ability of this ST to predominate and exhibit increased virulence.

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Transparency declaration

The authors declare no competing or financial interests in this work.

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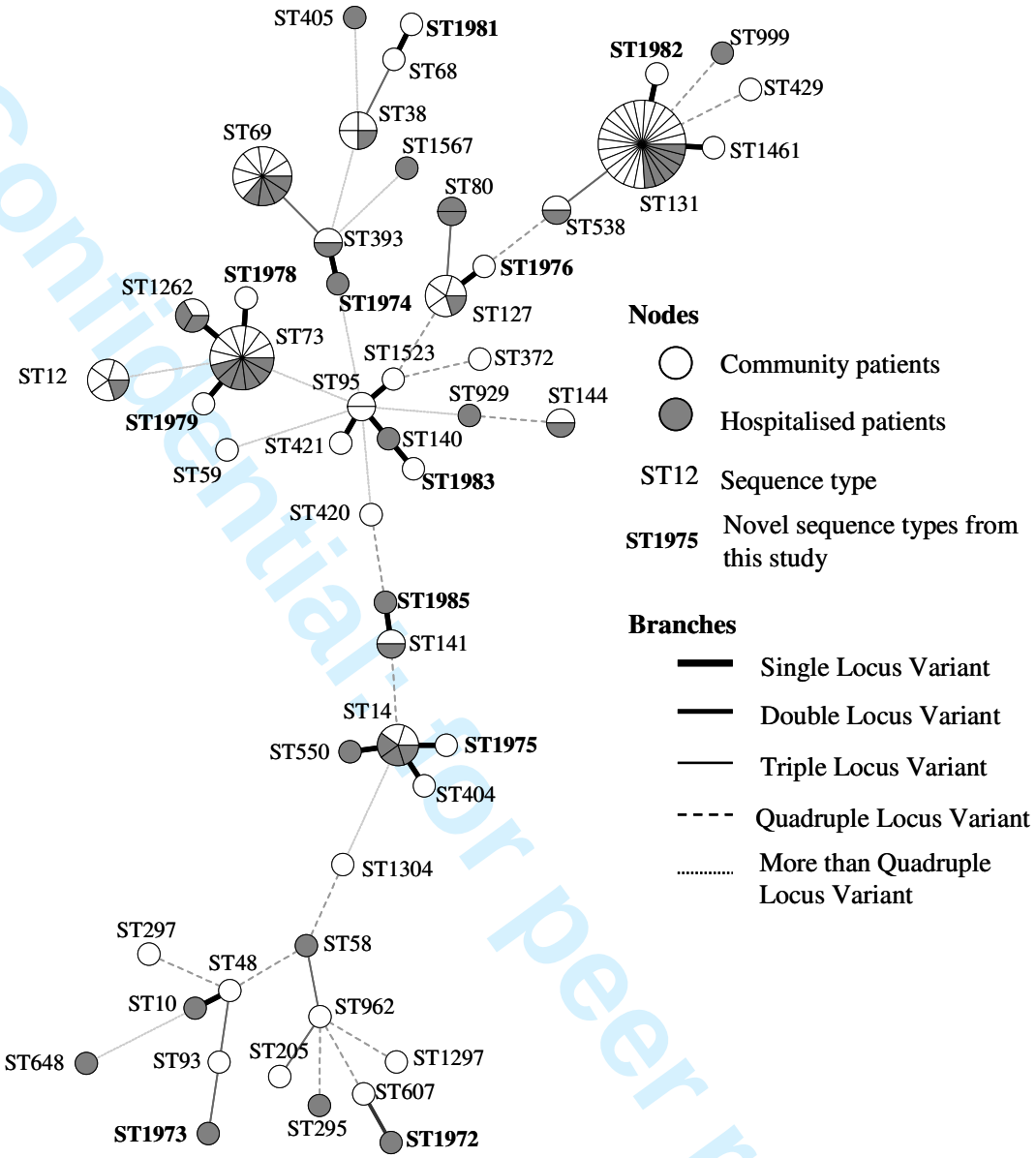
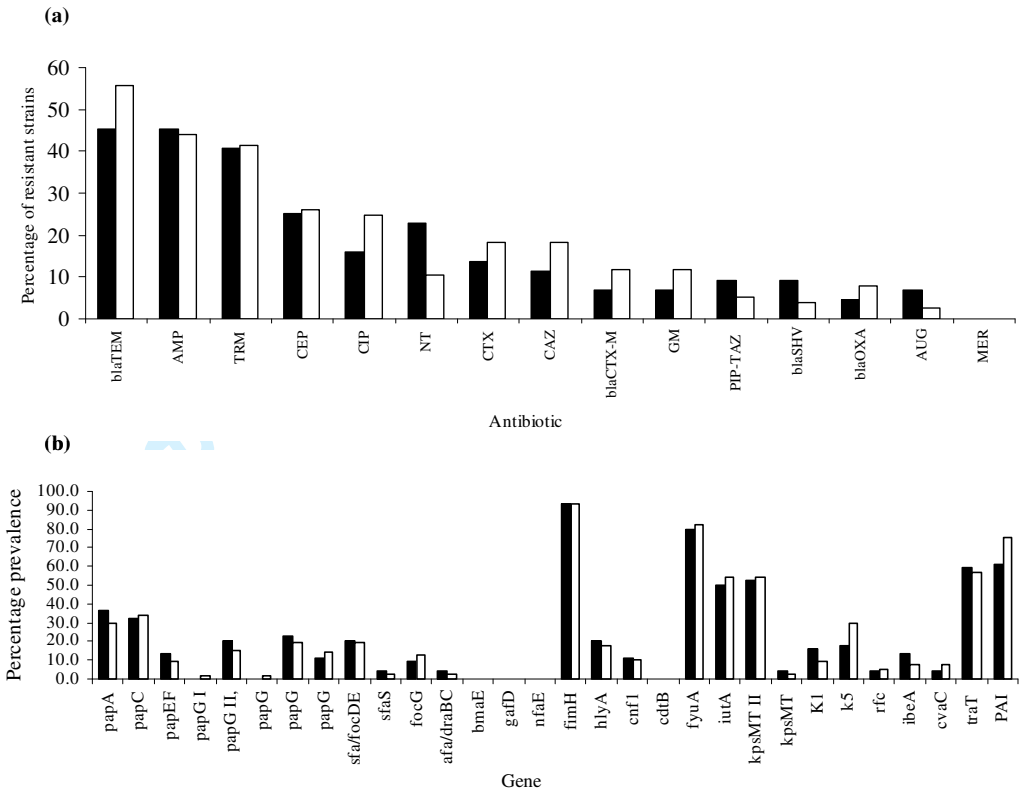


Fig. 1. Minimum spanning tree of *E. coli* sequence types from elderly urinary tract infections. Grey shading indicates proportion of isolates within ST derived from hospital environments and un-shaded areas indicate proportion of isolates within ST derived from community environments. Strains within the same ST possess 7 identical alleles, whereas a single locus variant possesses one different allele to the other ST and a double locus variant differs by two alleles.



380

381 **Fig. 2.** (a) Antibiotic resistance prevalence in urinary *E. coli* isolates of hospital and

382 community origin. Black bars, hospital isolates; white bars, community isolates. (b) Virulence

383 associated gene prevalence in urinary *E. coli* isolates of hospital and community origin. Black

384 bars, hospital isolates; white bars, community isolates.

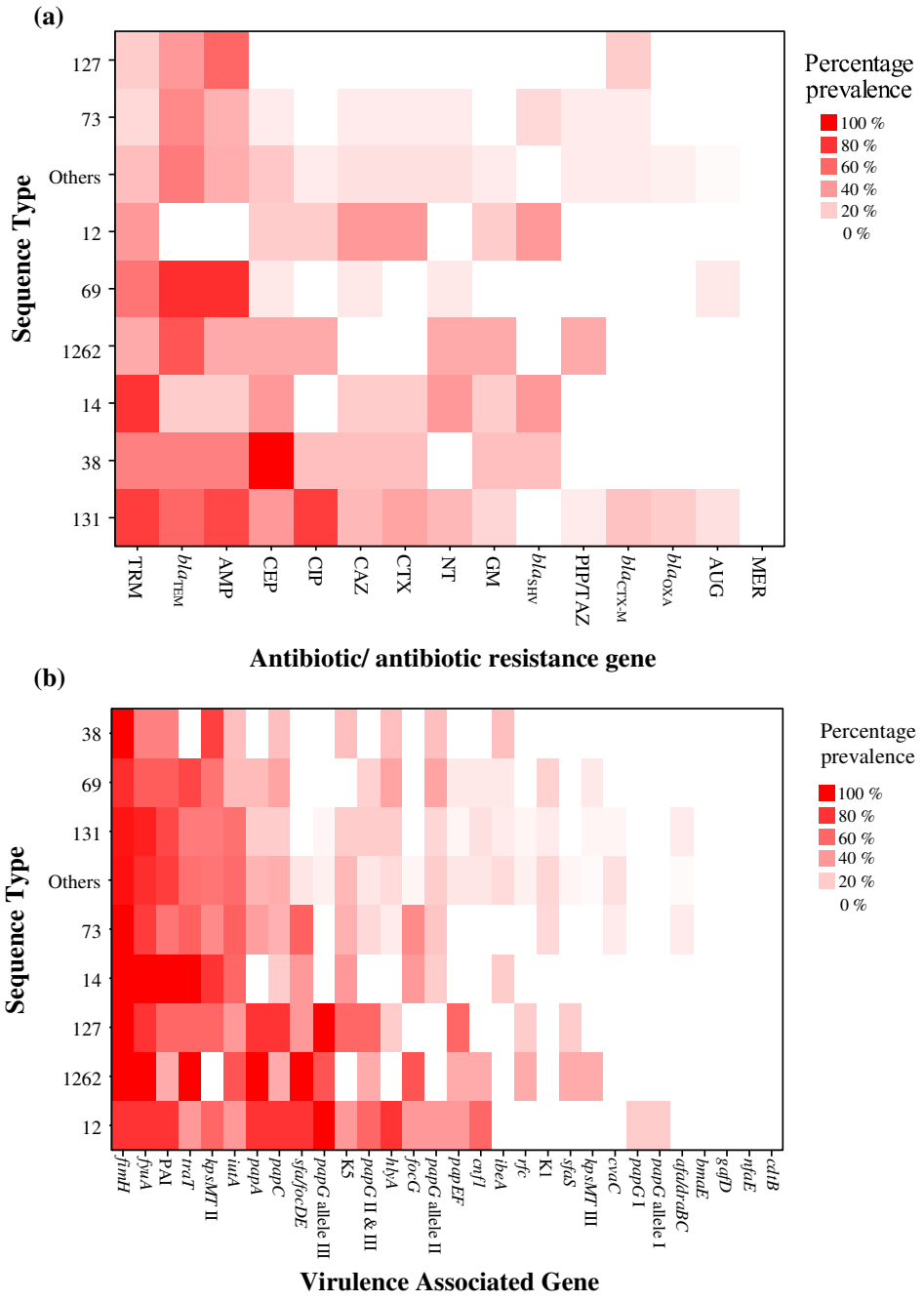
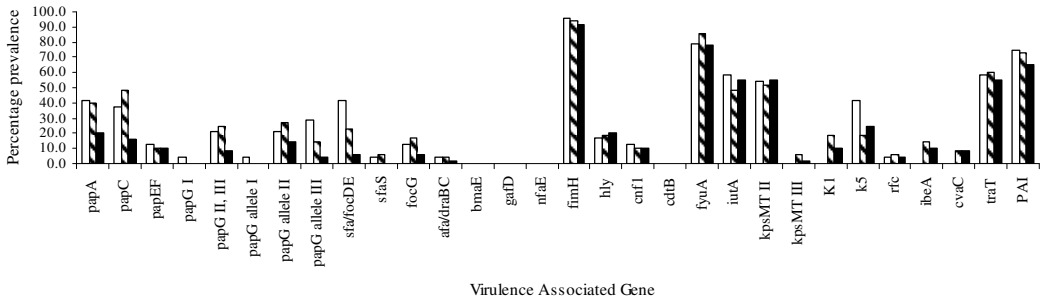


Fig. 3. (a) Heat map of antimicrobial resistance distribution across predominant *E. coli* sequence types. Darker shaded areas indicate higher prevalence. Abbreviations; bla_{TEM}, TEM β-lactamase gene; bla_{SHV}, SHV β-lactamase gene; bla_{CTX-M}, CTX-M β-lactamase gene; bla_{OXA}, OXA β-lactamase gene; GM, Gentamicin; CTX, Cefotaxime; CAZ, Ceftazidime; MER, Meropenem; PIP-TAZ, Piperacillin-tazobactam; AUG, Augmentin; TRM, Trimethoprim; CIP, Ciprofloxacin; CEP, Cephradine; NT, Nitrofurantoin; AMP, Ampicillin. (b) Heat map of virulence associated gene distribution across predominant *E. coli* sequence types.



393

394 **Fig. 4.** Virulence associated gene prevalence in *E. coli* isolates with different invasive

395 potentials. White bars, isolates with low invasive potential; hatched bars, strains with normal

396 invasive potential comparable to that of CFT073; black bars, strains with high invasive

397 potential.

398