

Increased human pathogenic potential of *Escherichia coli* from polymicrobial urinary tract infections in comparison to isolates from monomicrobial culture samples

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The current diagnostic standard procedure outlined by the Health Protection Agency for urinary tract infections (UTIs) in clinical laboratories does not report bacteria isolated from samples containing three or more different bacterial species. As a result many UTIs go unreported and untreated, particularly in elderly patients, where polymicrobial UTI samples are especially prevalent. This study reports the presence of the major uropathogenic species in mixed culture urine samples from elderly patients, and of resistance to front-line antibiotics, with potentially increased levels of resistance to ciprofloxacin and trimethoprim. Most importantly, the study highlights that *Escherichia coli* present in polymicrobial UTI samples are statistically more invasive ($P < 0.001$) in *in vitro* epithelial cell infection assays than those isolated from monomicrobial culture samples. In summary, the results of this study suggest that the current diagnostic standard procedure for polymicrobial UTI samples needs to be reassessed, and that *E. coli* present in polymicrobial UTI samples may pose an increased risk to human health.

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INTRODUCTION

The annual incidence of urinary tract infections (UTIs) in the elderly population ranges from 10% in the community to as high as 30% of hospitalized patients (Cove-Smith & Almond, 2007). Mortality rates in elderly patients from bacteraemia as a result of UTI can be as high as 33% (Tal *et al.*, 2005). A study by Plowman *et al.* (2001) found that over a 12 month period UTIs had the highest incidence (35%) of all nosocomial infections in a district general hospital, and the majority of patients were over 60 years of age. UTIs are also the most common infection in long-term care facilities, where they account for 20–60% of all antibiotic prescription use (Nicolle, 2001a). This large-scale prescription of antibiotics may well contribute to the levels of antibiotic resistance in urinary pathogens (Zhanel *et al.*, 2005). The main aetiological agent of UTIs is well documented as *Escherichia coli* (Nicolle, 2001b; Farajnia *et al.*, 2009; Kumazawa & Matsumoto, 1997). The establishment of urinary tract pathogens is thought to begin with the invasion of the superficial bladder epithelium, where bacteria

can form intracellular communities and receive a level of protection against the host immune system and antibiotic treatment (Anderson *et al.*, 2003; Blango & Mulvey, 2010).

The diagnosis of UTI is routinely made in the clinical laboratory by microbiological culture of a urine sample according to the national standard method developed and approved by the Health Protection Agency (HPA) (<http://www.hpa-standardmethods.org.uk/documents/bsop/pdf/bsop41.pdf>). Infections caused by a single organism are usually treatable with an antibiotic regimen advised on the basis of antibiotic susceptibility tests. However, it has been estimated that 33% of urine cultures from elderly patients are polymicrobial (Cove-Smith & Almond, 2007). Due to the difficulties of identifying the organisms present in these cultures, clinical microbiology laboratories do not report organisms isolated from urine in mixed cultures unless there is a significant count of a predominant organism.

As a result there is insufficient information on the bacteria that cause polymicrobial UTIs and the threat they pose to patient health. This study aimed to isolate the organisms present in polymicrobial UTI samples from a population of elderly patients, and compare their prevalence, phenotypic activity and pathogenic potential to monomicrobial culture isolates from the same population, with the aim of further understanding the potential threat posed by bacteria present in

Abbreviations: BSAC, British Society for Antimicrobial Chemotherapy; ESBL, extended-spectrum β -lactamase; ExPEC, extraintestinal pathogenic *Escherichia coli*; HPA, Health Protection Agency; MRSA, methicillin-resistant *Staphylococcus aureus*; NUH, Nottingham University Hospitals; PVL, Pantone–Valentine leukocidin; UTI, urinary tract infection.

polymicrobial urine samples from elderly patients, and possibly reassessing the current diagnostic standard procedure.

METHODS

Sample collection. A total of 250 urine culture agar plates were collected 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 patients aged 70 and over, and assigned to one of the following categories: (i) non-catheterized male polymicrobial infection, (ii) non-catheterized female polymicrobial infection, (iii) catheterized male polymicrobial infection, (iv) catheterized female polymicrobial infection. All of the categories contained cultures with three or more organisms and with no predominant count of one species. A fifth group, monomicrobial infection (uncatheterized or catheterized, males or females), was also included for use as a control group. Bacteria were isolated using standard microbiological identification procedures. Identification of isolates to the species level was performed using API identification systems, API 20E, API 20strep, API 20NE and API Staph (bioMérieux).

Antibiotic susceptibility testing. A breakpoint method was employed to obtain antibiotic susceptibility profiles for all *E. coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Proteus mirabilis*. The British Society for Antimicrobial Chemotherapy (BSAC) method for antimicrobial susceptibility testing was followed for preparing standardized inocula (Andrews, 2009). The antibiotic panel used was as follows: gentamicin (2 µg ml⁻¹), cefotaxime (1 µg ml⁻¹), ceftazidime (1 µg ml⁻¹), meropenem (2 µg ml⁻¹), piperacillin-tazobactam (16 µg ml⁻¹), co-amoxiclav (32 µg ml⁻¹), trimethoprim (2 µg ml⁻¹), ciprofloxacin (4 µg ml⁻¹), cefradine (32 µg ml⁻¹), nitrofurantoin (32 µg ml⁻¹) and amoxicillin (32 µg ml⁻¹). All *Pseudomonas aeruginosa* isolates were tested using the BSAC disc diffusion method (Andrews, 2009) and an antibiotic panel specific to *Pseudomonas*: gentamicin (10 µg ml⁻¹), piperacillin-tazobactam (85 µg ml⁻¹), ceftazidime (30 µg ml⁻¹), meropenem (10 µg ml⁻¹) and ciprofloxacin (1 µg ml⁻¹).

PCR detection of β-lactamase-encoding genes. All *E. coli*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Enterobacter* and *Citrobacter* isolates were tested for the presence of the β-lactamase-encoding genes, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{OXA} using a published multiplex PCR assay (Fang *et al.*, 2008). GenElute extraction kits (Sigma) were used to extract bacterial genomic DNA. Reference strains containing known β-lactamase types (NCTC 13351 *E. coli* *bla*_{TEM-3}, NCTC 13353 *E. coli* *bla*_{OXA}, *bla*_{CTX-M-15} and *bla*_{TEM}, and NCTC 13368 *Klebsiella pneumoniae* *bla*_{TEM}, *bla*_{SHV-18}) were included as controls.

Extended-spectrum β-lactamase (ESBL) confirmation tests. ESBL combination identification discs were used to confirm the extended-spectrum activity of selected β-lactamase-positive strains. The BSAC method for antimicrobial susceptibility testing (Andrews, 2009) was followed for preparing a standardized inoculum, which was used to inoculate an Iso-Sensitest agar plate to produce a lawn of growth. Two sets of combination discs were used to optimize ESBL detection: cefpodoxime/cefepime and clavulanic acid, and ceftazidime/cefepime and clavulanic acid. An increase in the diameter of the zone of inhibition of ≥5 mm for the cephalosporin/clavulanic acid disc compared to the cephalosporin alone disc indicated the presence of an ESBL-producing organism. Reference organisms *E. coli* NCTC 13351, *E. coli* NCTC 13352, *E. coli* NCTC 13353 and *E. coli* NCTC 10418 (HPA culture collections) were used for control purposes.

Detection of vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA) and Pantone-Valentine

leukocidin (PVL) producing *S. aureus*. A published protocol (Jayaratne & Rutherford, 1999) was used to screen all *Enterococcus faecalis* and *Enterococcus faecium* isolates for the presence of *vanA* and *vanB* genes. GenElute extraction kits (Sigma) were used to extract bacterial genomic DNA. *Enterococcus faecalis* NCTC 12201 was used as a reference strain.

A real-time PCR protocol developed by Thomas *et al.* (2007) was used to detect the presence of the *mecA* gene, which confers resistance to methicillin, in all *S. aureus* isolates. PCR was also performed for the detection of the PVL-encoding gene, *lukF*, using a published protocol (Ribeiro *et al.*, 2005). The BSAC disc diffusion protocol (Andrews, 2009) was followed to test all *S. aureus* isolates for phenotypic resistance to ceftazidime, which is indicative of methicillin resistance, and a selection of *Enterococcus* isolates for phenotypic resistance to vancomycin.

BOX-PCR. To confirm the organisms isolated from the UTI cultures were not all related to a single locally disseminated clone, BOX-PCR was performed using a protocol adapted from that of Koeuth *et al.* (1995). Extraintestinal pathogenic *E. coli* (ExPEC) CFT073 was used as a reference strain. PCR products were examined using BioNumerics v. 3.5 software (Applied Maths), using CFT073 to monitor the repeatability of the experiment.

Cell cultures. T24 human epithelial cells (HPA cultures) were grown in McCoy's 5A modified media (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 0.75% L-glutamine (Sigma). Cells were grown in an atmosphere with 5% CO₂ 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 *E. coli* assays were performed in duplicate on different days, and in triplicate wells in each assay. Bacteria were cultured overnight in Luria-Bertani broth, harvested by centrifugation and resuspended in supplemented tissue culture medium, which was then adjusted to 2 × 10⁷ c.f.u. ml⁻¹, giving an m.o.i. of 1 : 100. For *E. coli*, all 129 polymicrobial ExPEC and 21 monomicrobial ExPEC isolated in this study were quantifiably assayed using classical gentamicin protection assays as described previously (McNally *et al.*, 2007), using cultured T24 bladder cells. The invasive ExPEC type 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 the Miles and Misra plate count method 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.

All *Enterococcus faecalis*, *Proteus mirabilis*, *P. aeruginosa* and *S. aureus* were subjected to a semiquantitative screen performed in triplicate on different days to assess levels of invasion. Assays were completed using the above method, which was adapted for 96-well plates as described by Javed *et al.* (2010).

Statistical analysis. χ² tests were performed to compare invasion and antimicrobial resistance between the monomicrobial culture and mixed culture populations, and also bacterial prevalence in different patient groups.

RESULTS

Similar bacterial species are isolated from monomicrobial and polymicrobial UTI samples

Urine culture plates were collected from 250 patients over a 9 month period from NUH, and were taken from both

hospitalized and community patients, with the median age of patients being 83.5 years. Of the 200 polymicrobial cultures collected, 71 (35.5%) contained 2 organisms, 90 (45%) contained 3 organisms, 36 (18%) contained 4 organisms and 3 (1.5%) contained 5 organisms. A total of 83 patients (33%) had a previous history of UTI, 27 cases of which were designated 'mixed' by the NUH clinical laboratory. Of these 27 patients presenting with a polymicrobial UTI, 13 went on to have further incidences of polymicrobial UTI after this study, 5 patients went on to have a UTI sample with a confirmed organism in monomicrobial culture and 9 had no further infections.

A total of 621 bacterial strains were isolated from the 250 urine cultures and identified to the species level using API identification systems (Fig. 1). The most predominant organism in both catheterized and uncatheterized patients was *E. coli*, which was also far more prevalent in female patient cultures (83%) than male cultures (46%) ($P<0.001$). In male samples *Enterococcus faecalis* was equally as ubiquitous as *E. coli*. These two organisms were also frequently associated in mixed cultures (36%). *E. coli* was isolated from 68% of polymicrobial cultures and 48% of monomicrobial cultures, and *Enterococcus faecalis* was isolated from 55% of polymicrobial cultures, but was significantly less frequently isolated from monomicrobial cultures (8%) ($P<0.001$). *P. aeruginosa*, *Proteus mirabilis* and *S. aureus* were also frequently isolated from 23, 25 and 10.5% of polymicrobial cultures, respectively.

BOX-PCR profiles provide evidence of a genetically diverse population

In order to ensure that the organisms isolated were not epidemic clones, the clonal relatedness of *E. coli*, *Enterococcus faecalis*, *Proteus mirabilis*, *P. aeruginosa* and *S. aureus* isolates was determined by BOX-PCR. BOX-group

numbers were allocated based upon a similarity threshold of 92%, which was decided upon after reviewing other publications (Proudy *et al.*, 2008; Yang *et al.*, 2004). The *E. coli* strains exhibited varying BOX profiles (Fig. 2) and did not appear to belong to a dominant epidemic clone. Nineteen distinct clonal groups of *E. coli* were assigned, the largest of which encompassed 71 of the total 150 *E. coli* strains in the collection. Eleven distinct BOX groups of *Enterococcus faecalis* were detected, with the majority of isolates belonging to two of the groups, group 1 containing 53 isolates and group 3 containing 37 isolates. A total of 18 of the 51 *P. aeruginosa* isolates were deemed to belong to the same BOX group, and other isolates were also allocated to 11 other BOX groups. *Proteus mirabilis* were allocated to five BOX groups in total, with 88% of strains found to belong to a single BOX group. *S. aureus* were allocated to seven BOX groups with 50% of the total strains belonging to one group. These results suggest that the isolates are not derived from a single epidemic clone of each species, and are indeed individual strains that could reasonably be expected to possess varying phenotypic and genotypic properties.

Antibiotic resistance is comparable between bacteria from mixed and monomicrobial cultures

To determine the specific antibiotic resistances within the population of polymicrobial UTI organisms, 394 isolates of the 5 most commonly isolated species (*E. coli*, *Enterococcus faecalis*, *P. aeruginosa*, *Proteus mirabilis* and *S. aureus*) were subjected to antibiotic susceptibility tests using an antibiotic panel presently used in the NUH clinical laboratory (Table 1). The results suggest that bacteria isolated from mixed culture samples exhibit comparable levels of resistance to front-line antibiotics as that observed in isolates from monomicrobial culture samples, regardless of species. With respect to *E. coli*, the percentage of mixed culture sample isolates exhibiting resistance to ciprofloxacin and

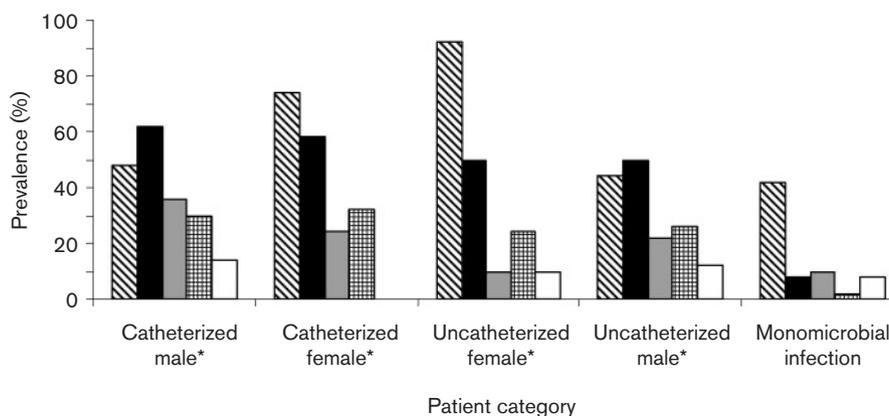


Fig. 1. The relative prevalence of each of the main species associated with UTI isolated from the different types of sample collected in the study. Prevalence is presented as the percentage of samples collected that contained the given species. Asterisks indicate cultures taken from polymicrobial infections. Hatched bars, *E. coli*; black bars, *Enterococcus faecalis*; grey bars, *P. aeruginosa*; cross-hatched bars, *Proteus mirabilis*; white bars, *S. aureus*.

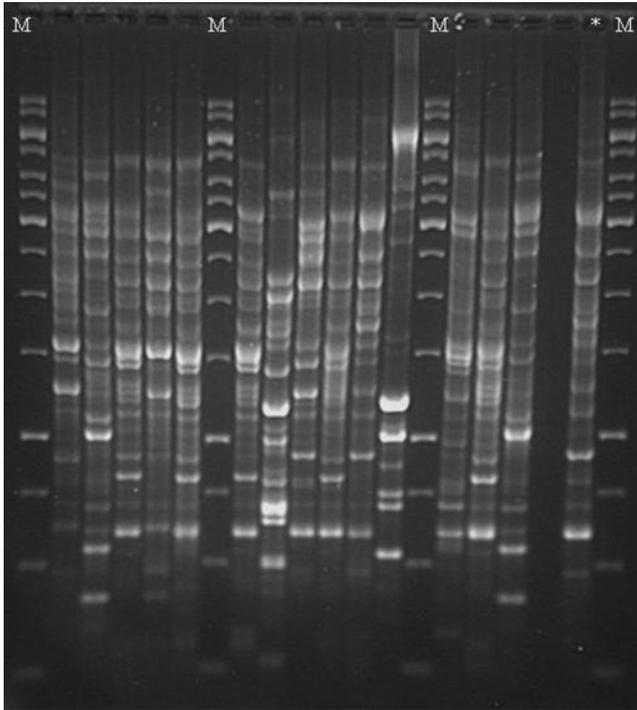


Fig. 2. BOX-PCR gel of *E. coli* strains isolated from UTI samples. The lane marked with an asterisk contains the BOX-PCR profile for *E. coli* CFT073; the remaining lanes show a selection of BOX profiles obtained for *E. coli* isolates. M, Molecular mass marker.

trimethoprim was higher than that observed in monomicrobial culture sample isolates, and also higher than the levels observed routinely in clinical UTI isolates. The differences observed in this study are not statistically significant but require further specific investigation.

Increased detection of ESBLs in monomicrobial culture isolates

Due to the increasing prevalence of ESBLs in clinical samples, the UTI isolates were screened both for the possession of β -lactamase-encoding genes and also for any ESBL phenotypic activity. All *E. coli*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus* and *Pseudomonas* strains ($n=355$) were screened for the presence of bla_{TEM} , bla_{SHV} , bla_{CTX-M} , and bla_{OXA} genes using a published protocol (Fang *et al.*, 2008). Forty-nine per cent of strains were found to possess a β -lactamase-encoding gene, and one fifth of the strains possessed multiple bla genes (Table 2). Interestingly most of the strains containing multiple β -lactamases originated from monomicrobial culture infections ($P=0.009$). *Klebsiella* species were found to possess the most β -lactamases (93% positive), 61% of *E. coli* also possessed a form of β -lactamase and 12% possessed multiple β -lactamase-encoding genes. The ESBL CTX-M was found significantly more frequently in monomicrobial culture isolates than in isolates from polymicrobial infections

($P=0.014$). To determine the phenotypic expression of extended-spectrum resistance against β -lactam antibiotics the double-disc method was used to screen all 173 β -lactamase PCR-positive strains. A total of 16 strains (9%) exhibited phenotypic ESBL activity (12 *E. coli*, 3 *Pseudomonas* and 1 *Enterobacter*), most of which was in strains that possessed multiple β -lactamase-encoding genes, making it impossible to identify the β -lactamase-encoding gene responsible for the extended-spectrum resistance.

Detection of MRSA and PVL-producing *S. aureus* in polymicrobial UTI samples

PCR detection of the *mecA* gene, confirmed 10 (45%) of the urinary *S. aureus* isolates to be MRSA. One of these was isolated from a monomicrobial culture UTI, and the remaining nine confirmed MRSA were isolated from polymicrobial UTI samples. Phenotypic resistance tests confirmed all PCR MRSA strains to be resistant to ceftazidime, which is an indicator of methicillin resistance. The *lukF* gene, which encodes PVL, was detected in two methicillin-sensitive *S. aureus* strains, which were both isolated from polymicrobial UTI samples. No vancomycin resistance genes were found in any of the *Enterococcus faecalis* isolates, but *vanA* was found in one *Enterococcus faecium* isolate. This isolate did not however show any phenotypic resistance to vancomycin.

Bacteria isolated from polymicrobial UTI samples exhibit increased pathogenic potential in *in vitro* cell invasion assays

The ability to invade host epithelial cells is a critical factor in UTI. Therefore, all *E. coli*, *Enterococcus faecalis*, *P. aeruginosa*, *Proteus mirabilis* and *S. aureus* strains were investigated to determine their ability to invade a human uroepithelial cell line. T24 human bladder cells were infected with a bacterial culture for 3 h, after which external bacteria were killed by the addition of gentamicin and internalized bacteria were enumerated. In the case of the *E. coli* assays, strains were designated highly invasive if they showed a 10-fold increase in invasiveness compared to that of the invasive reference strain, *E. coli* CFT073, which exhibited variation of less than 1 log across all assays performed (less than 10-fold). Overall 52 strains (34.7%) were seen to exhibit the highly invasive phenotype and 21 strains (14%) exhibited a low invasive phenotype, more than a 10-fold reduction in invasion compared to CFT073. *E. coli* isolates from mixed culture samples exhibited increases in invasion as great as 1000-fold higher than that observed in the invasive type strain CFT073. No invasive capacity was observed in five strains, of which three were isolated from monomicrobial culture samples where they were reported as the infectious agent. Overall 45% of the polymicrobial *E. coli* strains assayed invaded to a similar level (less than a 10-fold increase or decrease) as CFT073 (Fig. 3). This is not significantly different to the monomicrobial culture isolates, 62% of which showed similar invasion capability to CFT073. In contrast 44% of

Table 1. Prevalence of antibiotic resistance in UTI isolates

'Poly' refers to strains of polymicrobial infection origin, whilst 'mono' refers to strains of monomicrobial infection origin. –, Particular species/antibiotic combination not tested.

Antibiotic concentration ($\mu\text{g ml}^{-1}$)	<i>Escherichia coli</i> (%)		<i>Enterococcus faecalis</i> (%)		<i>Proteus mirabilis</i> (%)		<i>Staphylococcus aureus</i> (%)		<i>Pseudomonas aeruginosa</i> (%)	
	Poly (n=129)	Mono (n=21)	Poly (n=110)	Mono (n=4)	Poly (n=56)	Mono (n=1)	Poly (n=18)	Mono (n=4)	Poly (n=46)	Mono (n=5)
Gentamicin (2)	12.4	4.76	–	–	5.3	0	4.5	0	–	–
Cefotaxime (1)	17.8	14.29	–	–	33.9	100.0	–	–	–	–
Ceftazidime (1)	18.6	9.52	–	–	35.7	100.0	–	–	–	–
Meropenem (2)	0	0	15.5	0	0	0	–	–	–	–
Piperacillin–tazobactam (16)	6.2	4.76	4.5	25.0	23.2	100.0	–	–	–	–
Co-amoxiclav (32)	5.4	0	2.7	0	16.1	100.0	0	0	–	–
Trimethoprim (2)	44.2	28.57	–	–	89.0	0	22.2	0	–	–
Ciprofloxacin (4)	23.3	9.52	28.2	0	0	0	55.5	50.0	–	–
Cefradine (32)	28.7	19.05	–	–	55.3	100.0	50.0	25.0	–	–
Nitrofurantoin (32)	17.1	9.52	10.9	0	–	–	11.0	0	–	–
Amoxicillin (32)	45.0	42.86	7.3	0	37.5	100.0	66.6	75.0	–	–
Gentamicin (10)	–	–	–	–	–	–	–	–	2.17	0
Piperacillin–tazobactam (85)	–	–	–	–	–	–	–	–	0	0
Ceftazidime (30)	–	–	–	–	–	–	–	–	2.17	0
Meropenem (10)	–	–	–	–	–	–	–	–	2.17	0
Ciprofloxacin (1)	–	–	–	–	–	–	–	–	4.35	0

polymicrobial *E. coli* strains were highly invasive, whereas no monomicrobial culture isolates invaded to a greater level than CFT073 and 11 % of polymicrobial isolates were classified as 'low invasive' compared to 38 % of monomicrobial culture isolates. This suggests that *E. coli* isolated from polymicrobial UTI samples may be significantly more invasive ($P < 0.001$, χ^2 distribution of strains with high, normal and low invasive capacity across polymicrobial and monomicrobial populations) in an *in vitro* uroepithelial cell infection model, than *E. coli* isolated as monomicrobial cultures from UTI samples. No association could be found between invasiveness and patient gender, previous history of UTI, catheterization status, health status of the patient, antibiotic resistance profile or BOX-PCR group.

Invasion assays were also performed on all *Enterococcus faecalis*, *Proteus mirabilis*, *P. aeruginosa* and *S. aureus* isolates. A wide range in invasion capability was noted in *Enterococcus faecalis* isolates, ranging from 10^6 to 10^2 c.f.u. bacteria ml^{-1} recovered from invasion assays. *Proteus mirabilis*, *P. aeruginosa* and *S. aureus* showed a similar trend with a 3-log range in invasion. Similarly the increased/decreased invasion levels were not attributable to any patient characteristic, antibiotic resistance profile or BOX-PCR group.

DISCUSSION

UTI rates in elderly people in the community can be as high as 10 % at any one time, and this figure can rise to

30 % for hospitalized patients. Bacteraemic UTIs in elderly patients can result in sepsis and death (Cove-Smith & Almond, 2007). A clinical microbiology laboratory will not routinely pursue or report organisms present in mixed culture from urine samples unless there is a significant count of a predominant organism. As up to 33 % of samples can be polymicrobial a large proportion of infections from the elderly population may go untreated or indeed be treated with inappropriate antibiotics. This will only serve to encourage the development of antibiotic resistance in urinary pathogens.

The predominant organism in polymicrobial UTI samples was *E. coli*, which concurs with the general consensus among published data concerning monomicrobial culture UTIs (Farajnia *et al.*, 2009; Tal *et al.*, 2005; Johnson, 1991; Brzuszkiewicz *et al.*, 2006). *Enterococcus faecalis* was the second most commonly isolated organism, and was significantly more prevalent in polymicrobial cultures than monomicrobial cultures ($P < 0.001$). *Enterococcus faecalis* and *E. coli* were found together in 36 % of cultures, and co-infection by these two pathogens may pose important questions for the antibiotic treatment of polymicrobial UTI as *Enterococcus* is known to be intrinsically resistant to many antibiotics, including several first-choice antibiotics for the treatment of UTI. The presence of both *Enterococcus faecalis* and uropathogenic *E. coli* in an infection not only may create difficulties in devising an antibiotic treatment regimen but also may exacerbate the pathogenicity of the *E.*

Table 2. β -lactamase-encoding gene carriage in UTI isolates as determined by PCR

Strains tested were all *E. coli*, *Pseudomonas* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp. that were isolated in this study.

Carriage of β -lactamase-encoding gene	Percentage of strains
Any β-lactamase	
Monomicrobial culture isolates	57.1
Polymicrobial culture isolates	48.4
SHV	
Monomicrobial culture isolates	8.6
Polymicrobial culture isolates	9.8
TEM	
Monomicrobial culture isolates	45.7
Polymicrobial culture isolates	36.4
CTX-M	
Monomicrobial culture isolates	22.9*
Polymicrobial culture isolates	9.5
OXA	
Monomicrobial culture isolates	11.4
Polymicrobial culture isolates	4.7
Multiple β-lactamases	
Monomicrobial culture isolates	22.9†
Polymicrobial culture isolates	8.8

* $P=0.016$.

† $P=0.01$ (χ^2 test).

coli, as recent studies have suggested (Lavigne *et al.*, 2008; Montravers *et al.*, 1997).

Prescription of ineffective antibacterial agents can increase the selection pressure for antibiotic-resistant agents within an infection. Organisms present in polymicrobial UTI cultures, that would not routinely be investigated, possessed antibiotic resistance to front-line antibiotics such as trimethoprim,

ciprofloxacin and amoxicillin. More importantly, the percentage of mixed culture sample *E. coli* isolates exhibiting resistance to trimethoprim and ciprofloxacin was higher than that observed in monomicrobial culture sample isolates, and also higher than the levels observed routinely in clinical isolates. Another issue raising concerns for patient health is the presence of MRSA and PVL-producing methicillin-sensitive *S. aureus* in polymicrobial UTI samples, which would not be detected under the current guidelines for UTI diagnosis. The presence of MRSA in an infection limits the choice of antibiotics available for treatment, and the cytotoxin PVL attacks white blood cells and can cause severe tissue necrosis (Holmes *et al.*, 2005). Other specific antibiotic-resistance traits were also detected, such as ESBLs, by PCR for known β -lactamase-encoding genes, although only a small proportion showed phenotypic activity. This discrepancy re-emphasizes the importance of screening clinical isolates for ESBL gene carriage as opposed to phenotypic tests (Livermore & Hawkey, 2005; Tofield *et al.*, 2007; Xu *et al.*, 2005). Interestingly, the current increase in isolation of *E. coli* 025b-ST131 CTX-M^R from clinical samples (Lau *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008; Vincent *et al.*, 2010) does not appear to be reflected in this study, with only 11 % of *E. coli* containing the *bla*_{CTX-M} gene. The full genetic diversity and lineage of the ExPEC strains isolated in this study is the current focus of intensive research.

It has been suggested that the critical step in UTI initiation is the attachment to and invasion of the superficial bladder epithelium, especially in the case of *E. coli* (Anderson *et al.*, 2003; Mulvey *et al.*, 2001). By attaching to bladder epithelial cells *E. coli* are able to establish reservoirs known as intra-cellular bacterial communities, from which the invading bacteria receive some level of protection against the host immune system and also initiate recurrent infections. Therefore, invasive bacteria are considered more proficient in instigating an infection. Significant differences were identified in the invasive capabilities of

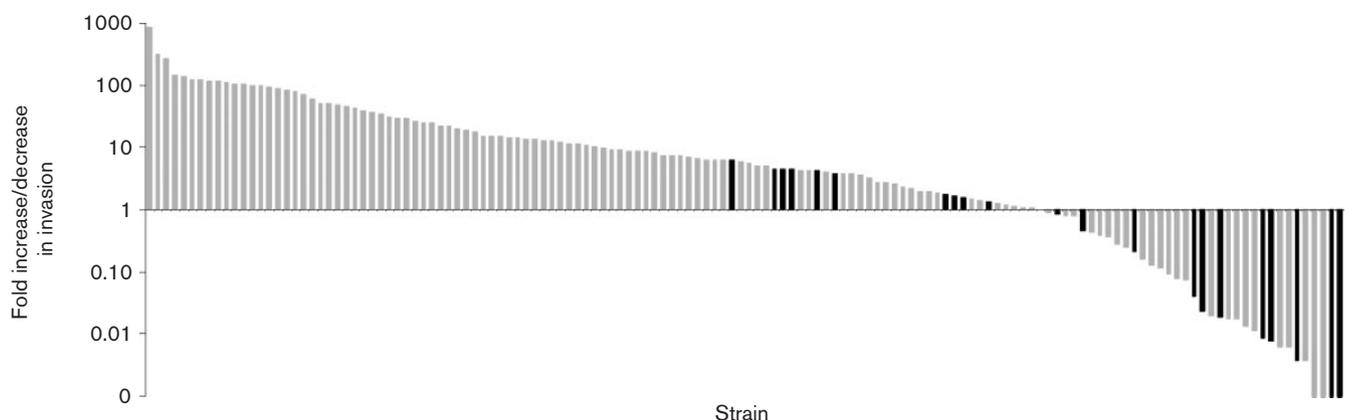


Fig. 3. Invasive capabilities of UTI *E. coli* isolates compared to *E. coli* CFT073. CFT073 invasion is designated 1. Polymicrobial sample isolates are statistically more invasive than monomicrobial culture sample isolates, $P<0.001$ (χ^2 test). Grey bars, polymicrobial culture strains; black bars, monomicrobial culture strains.

monomicrobial culture and polymicrobial culture isolates (Fig. 3). *E. coli* isolates of polymicrobial culture origin were significantly more invasive, when compared to the invasion of the type strain *E. coli* CFT073, than strains isolated from monomicrobial culture samples. The majority of isolates from monomicrobial infections were less invasive than *E. coli* CFT073 and none exhibited a highly invasive phenotype. The polymicrobial isolates that possessed increased invasive capacity did not belong exclusively to any specific patient group and were not shown to be associated with patient gender, catheterization status, previous history of UTI and underlying medical issues, antibiotic resistance profile or BOX-PCR group. This indicates the potential existence of a heterogeneous group of highly invasive *E. coli* within polymicrobial UTIs in the elderly, which would not be diagnosed or treated due to limitations in the current diagnostic standard procedure. A further study including larger numbers of ExPEC isolates from monomicrobial infections is required to rule out any such associations, and indeed to confirm the significance of the increased invasive phenotype exclusively observed in the polymicrobial isolates from this study. This is currently under investigation in several hospital laboratories examining equal numbers of polymicrobial and monomicrobial isolates, including an examination of the clinical nature of the infections, their association with complicated or uncomplicated UTI, and the genotypic and phenotypic differences associated with the hyper-invasive phenotype.

The frequent co-isolation of *E. coli* and *Enterococcus faecalis* from the clinical UTI samples raises questions as to the possible contribution of enterococci to the increased invasive phenotype expressed by the ExPEC strains isolated in this study. There have been reports of enterococci exacerbating the pathogenicity of other organisms including ExPEC in both *C. elegans* and rat models of infection (Lavigne *et al.*, 2008; Montravers *et al.*, 1997), and the promiscuous nature of enterococci with regard to gene transfer is well known. The possibility that enterococci can alter the genotype and/or phenotype of ExPEC during co-infection of bladder epithelial cells is currently the subject of further investigation.

Polymicrobial UTIs may pose a heightened threat to the health and well being of the elderly population. This study found that the organisms present in polymicrobial UTIs possess traits such as antibiotic resistance akin to that of their monomicrobial culture counterparts, and potentially with increased resistance to ciprofloxacin and trimethoprim, which are common front-line antibiotics used for UTI treatment. Due to the complexities involved in the diagnosis and treatment in these infections many patients may receive inadequate antibiotic treatment or indeed a lack of treatment altogether. More worryingly for patient health, the majority of organisms isolated from polymicrobial cultures also exhibited increased human pathogenic potential as evidenced by *in vitro* cell infection assays. The diagnostic standard procedure for UTI should be reconsidered in light of the data presented here.

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