

Increased Human Pathogenic Potential of *Escherichia coli* from Polymicrobial Urinary Tract Infections in Comparison to Isolates from Monomicrobial Culture Samples

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Running title: Pathogens in mixed culture UTI samples

1 **Abstract**

2 The current diagnostic standard procedure outlined by the HPA for urinary tract
3 infections (UTI) in clinical laboratories does not report bacteria isolated from
4 samples containing 3 or more different bacterial species. As a result many UTI go
5 unreported and untreated, particularly in elderly patients, where polymicrobial UTI
6 samples are especially prevalent. This study reports the presence of the major
7 uropathogenic species in mixed culture urine samples from elderly patients, and
8 of resistance to front line antibiotics, with potentially increased levels of
9 resistance to ciprofloxacin and trimethoprim. Most importantly, the study
10 highlights that *E. coli* present in polymicrobial UTI samples are statistically more
11 invasive ($P < 0.001$) in *in vitro* epithelial cell infection assays than those isolated
12 from monomicrobial culture samples. In summary this study suggests that the
13 current diagnostic standard procedure for polymicrobial UTI samples needs to be
14 re-assessed, and that *E. coli* present in polymicrobial UTI samples may pose an
15 increased risk to human health.

16

17 Key words: Urinary tract infection, polymicrobial, *E. coli*, antimicrobial, invasion

18

19 **Introduction**

20 The annual incidence of UTI in the elderly population ranges from 10% in the
21 community to as high as 30% of hospitalised patients (Cove-Smith & Almond,
22 2007). Mortality rates in elderly patients from bacteraemia as a result of UTI can
23 be as high as 33% (Tal *et al.*, 2005). A study by Plowman *et al.* (2001) found that
24 over a 12-month period urinary tract infections had the highest incidence (35%)
25 of all nosocomial infections in a district general hospital, and the majority of
26 patients were over 60 years of age. UTI are also the most common infection in
27 long term care facilities, where they account for 20-60% of all antibiotic
28 prescriptions (Nicolle, 2001a). This large-scale prescription of antibiotics may well
29 contribute to the levels of antibiotic resistance in urinary pathogens (Zhanel *et al.*,
30 2005). The main etiologic agent of UTI is well documented as *E. coli* (Nicolle *et*
31 *al.*, 2001b, Farajnia *et al.*, 2008, & Kumazawa & Matsumoto, 1997). The
32 establishment of urinary tract pathogens is thought to begin with the invasion of
33 the superficial bladder epithelium, where bacteria can form intracellular
34 communities and receive a level of protection against the host immune system
35 and antibiotic treatment (Anderson *et al.*, 2003, Blango & Mulvey, 2010).

36 The diagnosis of UTI is routinely performed in the clinical laboratory by
37 microbiological culture of a urine sample according to the national standard
38 method developed and approved by the Health protection Agency
39 (<http://www.hpa-standardmethods.org.uk/documents/bsop/pdf/bsop41.pdf>).

40 Infections caused by a single organism are usually treatable with an antibiotic
41 regimen advised on the basis of antibiotic susceptibility tests. However, it has
42 been estimated that 33% of urine cultures from elderly patients are polymicrobial

43 (Cove-Smith & Almond, 2007). Due to the difficulties of identifying the organisms
44 present in these cultures clinical microbiology laboratories do not report
45 organisms isolated from urine in mixed culture unless there is a significant count
46 of a predominant organism

47 As a result there is insufficient information on the bacteria that cause
48 polymicrobial UTI and the threat they pose to patient health. This study aimed to
49 isolate the organisms present in polymicrobial urinary tract infection samples
50 from a population of elderly patients, and compare their prevalence, phenotypic
51 activity and pathogenic potential to monomicrobial culture isolates from the same
52 population, with the aim of further understanding the potential threat posed by
53 bacteria present in polymicrobial urine samples from elderly patients, and
54 possibly reassessing the current diagnostic standard procedure.

55

56 **Materials and Methods**

57

58 **Sample collection**

59 Two hundred and fifty urine culture agar plates were collected from Nottingham
60 University Hospitals (NUH) between October 2008 and June 2009. Cultures were
61 collected anonymously (therefore no ethical approval or informed consent
62 required) from patients aged 70 and over and assigned to one of the following
63 categories; (1) non-catheterized male polymicrobial infection, (2) non-
64 catheterized female polymicrobial infection, (3) catheterized male polymicrobial
65 infection, (4) catheterized female polymicrobial infection, all of which contained 3
66 or more organisms with no predominant count of one species. A fifth group,

67 monomicrobial infection (uncatheterized or catheterized, males or females), was
68 also included for use as a control group. Bacteria were isolated using standard
69 microbiological identification procedures. Identification of isolates to species level
70 was performed using the API identification systems, API 20E, API 20strep, API
71 20NE and API Staph (Biomérieux,).

72 **Antibiotic susceptibility testing**

73 A breakpoint method was employed to obtain antibiotic susceptibility profiles for
74 all *E. coli*, *S. aureus*, *E. faecalis* and *P. mirabilis*. The BSAC method for
75 antimicrobial susceptibility testing was followed to prepare standardised inocula
76 (Andrews, 2009). The antibiotic panel used was as follows; Gentamicin (2µg/ml),
77 Cefotaxime (1 µg/ml), Ceftazidime (1 µg/ml), Meropenem (2 µg/ml), Piperacillin-
78 tazobactam (16 µg/ml), Co-amoxiclav (32 µg/ml), Trimethoprim (2 µg/ml),
79 Ciprofloxacin (4 µg/ml), Cephradine (32 µg/ml), Nitrofurantoin (32 µg/ml), and
80 Amoxicillin (32 µg/ml). All *P. aeruginosa* isolates were tested using the BSAC
81 disc diffusion method (Andrews, 2009) and an antibiotic panel specific to
82 *Pseudomonas*; Gentamicin (10µg/ml), Piperacillin-tazobactam (85 µg/ml),
83 Ceftazidime (30 µg/ml), Meropenem (10 µg/ml) and Ciprofloxacin (1 µg/ml).

84 **PCR detection of β-lactamase genes**

85 All *E. coli*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Enterobacter* and *Citrobacter*
86 isolates were tested for the presence of the β-lactamase genes, *bla*_{TEM}, *bla*_{SHV},
87 *bla*_{CTX-M} and *bla*_{OXA} using a previously described multiplex PCR (Fang *et al.*,
88 2008). GenElute extraction kits (SIGMA) were used to extract bacterial genomic
89 DNA. Reference strains containing known β-lactamase types (NCTC 13351 *E.*

90 *coli bla*_{TEM-3}, NCTC 13353 *E. coli bla*_{OXA}, *bla*_{CTX-M-15}, and *bla*_{TEM} and NCTC 13368
91 *Klebsiella pneumoniae bla*_{TEM}, *bla*_{SHV-18}) were included as controls.

92 **ESBL confirmation tests**

93 ESBL combination ID discs were used to confirm the extended spectrum activity
94 of selected β -lactamase positive strains. The BSAC method for antimicrobial
95 susceptibility testing (Andrews, 2009) was followed to prepare a standardised
96 inoculum, which was used to inoculate an Iso-Sensitest agar plate to produce a
97 lawn of growth. Two sets of combination discs were used to optimise ESBL
98 detection, Cefpodoxime/Cefpodoxime&clavulanate and
99 Cefepime/Cefepime&clavulanate. An increase in zone of inhibition diameter of
100 ≥ 5 mm of the cephalosporin/clavulanic acid disc compared to the cephalosporin
101 alone indicated the presence of an ESBL-producing organism. Reference
102 organisms *E. coli* NCTC 13351, *E. coli* NCTC 13352 and *E. coli* NCTC 13353
103 and *E. coli* NCTC 10418 (HPA culture collections) were used for control
104 purposes.

105 **Detection of vancomycin resistant enterococci, MRSA and Panton-** 106 **Valentine Leukocidin producing *S. aureus***

107 A previously published protocol, (Jayaratne *et al.*, 1999) was used to screen all
108 *E. faecalis* and *E. faecium* isolates for the presence of *vanA* and *vanB* genes.
109 GenElute extraction kits (SIGMA) were used to extract bacterial genomic DNA. *E.*
110 *faecalis* NCTC 12201 was used as a reference strain.

111 A real-time PCR protocol developed by Thomas *et al.*, (2007) was used to detect
112 the presence of the *mecA* gene, which confers resistance to methicillin, in all
113 *Staphylococcus aureus* isolates. PCR was also performed for the detection of the

114 PVL-encoding gene, *lukF*, using a previously published protocol (Ribeiro *et al.*,
115 2005). The BSAC disc diffusion protocol (Andrews, 2009) was followed to test all
116 *S. aureus* isolates for phenotypic resistance to ceftiofur, which is indicative of
117 methicillin resistance, and a selection of *Enterococcus* isolates for phenotypic
118 resistance to vancomycin.

119 **BOX-PCR**

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

126 **Cell cultures**

127 T24 human epithelial cells (HPA cultures) were grown in McCoy's 5A modified
128 media (Sigma, UK) supplemented with 10% fetal bovine serum (Sigma, UK) and
129 0.75% L-glutamine (Sigma, UK). Cells were grown in an atmosphere with 5%
130 CO₂ at 37°C and sub-cultured twice-weekly. Two days prior to cell infection
131 assays, the T24 cells were seeded into 24-well plates

132 **Association and invasion assays**

133 All *E. coli* assays were performed in duplicate on different days, and in triplicate
134 wells in each assay. Bacteria were cultured overnight in LB broth, harvested by
135 centrifugation and re-suspended in supplemented tissue culture medium, which
136 was then adjusted to 2×10^7 cfu/ml, giving an MOI of 1:100. For *E. coli*, all
137 polymicrobial ExPEC and 21 monomicrobial ExPEC isolated in this study were

138 quantifiably assayed using classical gentamicin protection assays as described
139 previously (McNally *et al.*, 2007), using cultured T24 bladder cells. The invasive
140 ExPEC type strain, CFT073 was used as a positive control strain in all assays
141 and *E.coli* DH5 α was used as a negative control strain. The mean number of
142 invasive bacteria was determined by Miles & Misra plate counts from triplicate
143 wells. Strains that showed more than a 10-fold increase in invasion compared to
144 CFT073 were classed as highly-invasive strains. Those that showed more than a
145 10-fold decrease in invasion compared to CFT073 were deemed to be strains of
146 limited invasive potential.

147 All *E. faecalis*, *P. mirabilis*, *P. aeruginosa* and *S. aureus* were subjected to a
148 semi-quantitative screen performed in triplicate on different days to assess levels
149 of invasion. Assays were completed using the above method, which was adapted
150 for 96-well plates as described by Javed *et al*, (2009).

151 **Statistical analysis**

152 χ^2 tests were performed to compare invasion and antimicrobial resistance
153 between the monomicrobial culture and mixed culture populations, and also
154 bacterial prevalence in different patient groups.

155

156 **Results**

157 **Similar bacterial species are isolated from monomicrobial and** 158 **polymicrobial UTI samples**

159 Urine culture plates were collected from 250 patients over a 9-month period from
160 Nottingham University Hospitals (NUH) and were taken from both hospitalised
161 and community patients, with the median age of patients being 83.5 years. Of the

162 200 polymicrobial cultures collected, 71 (36%) contained 2 organisms, 90 (45%)
163 contained 3 organisms, 36 (18%) contained 4 organisms and 3 (1%) contained 5
164 organisms. Eighty three patients (33%) had previous history of UTI, 27 cases of
165 which were designated as 'mixed' by the NUH clinical laboratory. Of these 27
166 patients presenting with a polymicrobial UTI, 13 went on to have further
167 incidences of polymicrobial UTI after this study, 5 patients went on to have a UTI
168 sample with a confirmed organism in monomicrobial culture and 9 had no further
169 infections.

170 A total of 620 bacterial strains were isolated from the 250 urine cultures and
171 identified to species level using API identification systems (figure 1). The most
172 predominant organism in both catheterised and uncatheterised patients was *E.*
173 *coli*, which was also far more prevalent in female patient cultures (83%) than
174 male cultures (46%) $P < 0.001$. In male samples *E. faecalis* was equally as
175 ubiquitous as *E. coli*. These two organisms were also frequently associated in
176 mixed cultures (36%). *E. coli* was isolated from 68% of polymicrobial cultures and
177 48% of monomicrobial cultures, and *E. faecalis* was isolated from 55% of
178 polymicrobial cultures, but was significantly less frequently isolated from
179 monomicrobial cultures (8%), $P < 0.001$. *P. aeruginosa*, *P. mirabilis* and *S. aureus*
180 were also frequently isolated from 23%, 25% and 10.5% of polymicrobial cultures
181 respectively.

182 **BOX-PCR profiles provide evidence of a genetically diverse population**

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

186 based upon a similarity threshold of 92% which was decided upon after reviewing
187 previous publications (Proudy *et al.*, 2008, & Yang *et al.*, 2004). The *E. coli*
188 strains exhibited varying BOX-profiles (figure 2) and did not appear to belong to a
189 dominant epidemic clone. Nineteen distinct clonal groups of *E. coli* were
190 assigned, the largest of which encompassed 71 of the total 150 *E. coli* strains in
191 the collection. Eleven distinct BOX-groups of *E. faecalis* were detected, with the
192 majority of isolates belonging to 2 of the groups, group 1 containing 53 isolates
193 and group 3 containing 37 isolates. Eighteen of the 51 *P. aeruginosa* isolates
194 were deemed to belong to the same BOX-group, and other isolates were also
195 allocated to 11 other BOX-groups. *P. mirabilis* were allocated 5 BOX-groups in
196 total, with 88% of strains found to belong to a single BOX-group. *S. aureus* were
197 allocated to 7 BOX-groups with 50% of the total strains belonging to one group.
198 These results suggest that the isolates are not derived from a single epidemic
199 clone of each species, and are indeed individual strains which could reasonably
200 be expected to possess varying phenotypic and genotypic properties.

201 **Antibiotic resistance is comparable between bacteria from mixed and**
202 **monomicrobial cultures**

203 To determine the specific antibiotic resistances within the population of
204 polymicrobial UTI organisms, 394 isolates of the 5 most commonly isolated
205 species (*E. coli*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis* and *S. aureus*) were
206 subjected to antibiotic susceptibility tests using an antibiotic panel presently used
207 in the NUH clinical laboratory (table 1). Results suggest that bacteria isolated
208 from mixed culture samples exhibit comparable levels of resistance to front line
209 antibiotics as that observed in isolates from monomicrobial culture samples,

210 regardless of species. With respect to *E. coli*, the percentage of mixed culture
211 sample isolates exhibiting resistance to ciprofloxacin and trimethoprim was
212 higher than that observed in monomicrobial culture sample isolates, and also
213 higher than the levels observed routinely in clinical urinary tract infection isolates.
214 The differences observed in this study are not statistically significant but require
215 further specific investigation.

216 **Increased detection of Extended Spectrum β -Lactamases in monomicrobial** 217 **culture isolates**

218 Due to the increasing prevalence of extended-spectrum β -lactamases (ESBLs) in
219 clinical samples the UTI isolates were screened both for the possession of β -
220 lactamase genes and also for any ESBL phenotypic activity. All *E. coli*,
221 *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus* and *Pseudomonas* strains (n=355)
222 were screened for the presence of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{OXA} genes
223 using a previously published protocol (Fang *et al.*, 2008). Forty-nine percent of
224 strains were found to possess a β -lactamase gene, and one fifth of the strains
225 possessed multiple *bla* genes (table 2). Interestingly most of the strains
226 containing multiple β -lactamases originated from monomicrobial culture
227 infections (P=0.009). *Klebsiella* species were found to possess the most β -
228 lactamases (93% positive), 61% of *E. coli* also possessed a form of β -lactamase
229 and 12% possessed multiple β -lactamase genes. The extended spectrum β -
230 lactamase, CTX-M was found significantly more frequently in monomicrobial
231 culture isolates than in isolates from polymicrobial infections (P=0.014). To
232 determine phenotypic expression of extended spectrum resistance against β -
233 lactam antibiotics the double-disc method was used to screen all 173 β -

234 lactamase PCR positive strains. Sixteen strains (9%) exhibited phenotypic ESBL
235 activity (12 *E. coli*, 3 *Pseudomonas* and 1 *Enterobacter*), most of which was in
236 strains that possessed multiple β -lactamase genes, making it impossible to
237 identify the β -lactamase gene responsible for the extended spectrum resistance.

238 **Detection of MRSA and PVL-producing *S. aureus* in polymicrobial UTI** 239 **samples**

240 PCR detection of the *mecA* gene, confirmed 10 (45%) of the urinary *S. aureus*
241 isolates to be MRSA. One of these was isolated from a monomicrobial culture
242 UTI, and the remaining 9 confirmed MRSA were isolated from polymicrobial UTI
243 samples. Phenotypic resistance tests confirmed all PCR MRSA strains to be
244 resistant to ceftazidime, which is an indicator of methicillin resistance. The *lukF*
245 gene, which encodes PVL production was detected in 2 methicillin sensitive *S.*
246 *aureus* (MSSA) strains, which were both isolated from polymicrobial UTI
247 samples. No vancomycin resistance genes were found in any of the *E. faecalis*
248 isolates, but *vanA* was found in one *E. faecium* isolate. This isolate did not
249 however show any phenotypic resistance to vancomycin.

250 **Bacteria isolated from polymicrobial UTI samples exhibit increased** 251 **pathogenic potential in *in vitro* cell invasion assays.**

252 The ability to invade host epithelial cells is a critical factor in UTI. Therefore all *E.*
253 *coli*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis* and *S. aureus* strains were
254 investigated to determine their ability to invade a human uroepithelial cell line.
255 T24 human bladder cells were infected with a bacterial culture for 3 hours, after
256 which external bacteria were killed by the addition of gentamicin and internalised
257 bacteria were enumerated. In the case of the *E. coli* assays, strains were

258 designated as highly invasive if they showed a 10-fold increase in invasiveness
259 compared to that of the invasive reference strain, *E. coli* CFT073, which exhibited
260 variation of less than 1 log across all assays performed (less than 10 fold).
261 Overall 52 strains (34.7%) were seen to exhibit the highly invasive phenotype
262 and 21 strains (14%) exhibited a low invasive phenotype, more than a 10 fold
263 reduction in invasion compared to that of CFT073. *E. coli* isolates from mixed
264 culture samples exhibited increases in invasion as great as 1000 fold higher than
265 that observed in the invasive type strain CFT073. No invasive capacity was
266 observed in five strains, of which three were isolated from monomicrobial culture
267 samples where they were reported as the infectious agent. Overall 45% of the
268 polymicrobial *E. coli* strains assayed invaded to a similar level (less than a ten-
269 fold increase or decrease) as that of CFT073 (figure 3). This is not significantly
270 different to the monomicrobial culture isolates, 62% of which showed similar
271 invasion capability to CFT073. In contrast 44% of polymicrobial *E. coli* strains
272 were highly invasive, whereas no monomicrobial culture isolates invaded to a
273 greater level than CFT073 and 11% of polymicrobial isolates were classified as
274 'low invasive' compared to 38% of monomicrobial culture isolates. This suggests
275 that *E. coli* isolated from polymicrobial UTI samples may be significantly more
276 invasive ($P < 0.001$, χ^2 distribution of strains with high, normal, and low invasive
277 capacity across polymicrobial and monomicrobial populations) in an *in vitro*
278 uroepithelial cell infection model, than *E. coli* isolated as monomicrobial cultures
279 from UTI samples. No association could be found between invasiveness and
280 patient gender, previous history of UTI, catheterisation status, health status of
281 patient, antibiotic resistance profile or BOX-PCR group.

282 Invasion assays were also performed on all *E. faecalis*, *P. mirabilis*, *P.*
283 *aeruginosa* and *S. aureus* isolates. A wide range in invasion capability was noted
284 in *E. faecalis* isolates, ranging from 10⁶cfu/ml to 10²cfu/ml bacteria recovered
285 from invasion assays. *P. mirabilis*, *P. aeruginosa* and *S. aureus* showed a similar
286 trend with a 3-log range in invasion. Similarly the increased/decreased invasion
287 levels are not attributable to any patient characteristic, antibiotic resistance profile
288 or BOX-PCR group.

289

290 **Discussion**

291 Urinary tract infection rates in elderly people in the community can be as high as
292 10% at any one time, and this figure can rise to 30% of hospitalised patients.
293 Bacteraemic UTI in elderly patients can result in sepsis and death (Cove-Smith &
294 Almond, 2007). A clinical microbiology laboratory will not routinely pursue or
295 report organisms present in mixed culture from urine samples unless there is a
296 significant count of a predominant organism. As up to 33% of samples can be
297 polymicrobial a large proportion of infections from the elderly population may go
298 untreated or indeed be treated with inappropriate antibiotics. This will only serve
299 to encourage the development of antibiotic resistance in urinary pathogens.

300 The predominant organism in polymicrobial UTI samples was *E. coli*, which
301 concurs with the general consensus among previously published data concerning
302 monomicrobial culture UTI (Farajnia *et al.*, 2008, Tal *et al.*, 2005, Johnson, 1991,
303 & Brzuszkiewicz *et al.*, 2006). *E. faecalis* was the second most commonly
304 isolated organism, and was significantly more prevalent in polymicrobial cultures
305 than monomicrobial cultures (P<0.001). *E. faecalis* and *E. coli* were found

306 together in 36% of cultures and co-infection by these two pathogens may pose
307 important questions for the antibiotic treatment of polymicrobial UTI as
308 *Enterococcus* is known to be intrinsically resistant to many antibiotics, including
309 several first choice antibiotics for the treatment of UTI. The presence of both this
310 organism and uropathogenic *E. coli* in an infection may not only create difficulties
311 in devising an antibiotic treatment regimen but also recent studies have
312 suggested *E. faecalis* may exacerbate the pathogenicity of *E. coli* (Lavigne *et al.*,
313 2008, & Montravers *et al.*, 1997).

314 Prescription of ineffective antibacterial agents can increase selection pressure for
315 antibiotic resistant agents within an infection. Organisms present in polymicrobial
316 UTI cultures, that would not routinely be investigated, possessed antibiotic
317 resistance to front line antibiotics such as trimethoprim, ciprofloxacin and
318 amoxicillin. More importantly the percentage of mixed culture sample *E. coli*
319 isolates exhibiting resistance to trimethoprim and ciprofloxacin was higher than
320 that observed in monomicrobial culture sample isolates, and also higher than the
321 levels observed routinely in clinical isolates. Another issue raising concerns for
322 patient health is the presence of MRSA and PVL-producing MSSA in
323 polymicrobial UTI samples, which would not be detected under the current
324 guidelines for UTI diagnosis. The presence of MRSA in an infection limits the
325 choice of antibiotics available for treatment, and the cytotoxin PVL attacks white
326 blood cells and can cause severe tissue necrosis (Holmes *et al.*, 2005). Other
327 specific antibiotic resistance traits such as extended-spectrum β -lactamases
328 were also detected, by PCR for known β -lactamase genes, although only a small
329 proportion showed phenotypic activity. This discrepancy re-emphasises the

330 importance of screening clinical isolates for ESBL gene carriage as opposed to
331 phenotypic tests (Livermore & Hawkey, 2005, Tofteland *et al.*, 2007, & Xu *et al.*,
332 2005). Interestingly, the current increase in isolation of *E. coli* 025b-ST131 CTX-
333 M^R from clinical samples (Lau *et al.*, 2008, Nicolas-Chanoine *et al.*, 2008, &
334 Vincent *et al.*, 2010) does not appear to be reflected in this study, with only 11%
335 of *E. coli* containing the *bla*_{CTX-M} gene. The full genetic diversity and lineage of the
336 ExPEC strains isolated in this study is the current focus of intensive research.
337 It has been suggested that the critical step in UTI initiation is the attachment to
338 and invasion of the superficial bladder epithelium, especially in the case of *E. coli*
339 (Anderson *et al.*, 2003, & Mulvey *et al.*, 2001). By attaching to bladder epithelial
340 cells *E. coli* are able to establish reservoirs known as intra-cellular bacterial
341 communities, from which the invading bacteria receive some level of protection
342 against the host immune system and also initiate recurrent infections. Therefore
343 invasive bacteria are considered more proficient in instigating an infection.
344 Significant differences were identified in the invasive capabilities of
345 monomicrobial culture and polymicrobial culture isolates (figure 3). *E. coli* isolates
346 of polymicrobial culture origin were significantly more invasive when compared to
347 the invasion of the type strain, *E. coli* CFT073, than strains isolated from
348 monomicrobial culture samples. The majority of isolates from monomicrobial
349 infections were less invasive than *E. coli* CFT073 and none exhibited a highly
350 invasive phenotype. The polymicrobial isolates that possessed increased
351 invasive capacity did not belong exclusively to any specific patient group and
352 were not shown to be associated with patient gender, catheterisation status,
353 previous history of UTI and underlying medical issues, antibiotic resistance profile

354 or BOX-PCR group. This indicates the potential existence of a heterogeneous
355 group of highly invasive *E. coli* within polymicrobial UTI in the elderly, which
356 would not be diagnosed or treated due to limitations in the current diagnostic
357 standard procedure. A further study including larger numbers of ExPEC isolates
358 from monomicrobial infections is required to rule out any such associations, and
359 indeed to confirm the significance of the increased invasive phenotype
360 exclusively observed in the polymicrobial isolates from this study. This is
361 currently under investigation in several hospital labs examining equal numbers of
362 polymicrobial and monomicrobial isolates, including an examination of the clinical
363 nature of the infections, their association with complicated or uncomplicated UTI,
364 and the genotypic and phenotypic differences associated with the hyper-invasive
365 phenotype.

366 The frequent co-isolation of *E. coli* and *E. faecalis* from the clinical UTI samples
367 raises questions as to the possible contribution of Enterococci to the increased
368 invasive phenotype expressed by the ExPEC strains isolated in this study. There
369 have been reports of Enterococci exacerbating the pathogenicity of other
370 organisms including ExPEC in both *C. elegans* and rat models of infection
371 (Lavigne *et al.*, 2008, & Montravers *et al.*, 1997), and the promiscuous nature of
372 Enterococci with regards to gene transfer is well known. The possibility that
373 Enterococci can alter the genotype and/or phenotype of ExPEC during co-
374 infection of bladder epithelial cells is currently the subject of further investigation.
375 Polymicrobial urinary tract infections may pose a heightened threat to the health
376 and well being of the elderly population. This study found that the organisms
377 present in polymicrobial UTI possess traits such as antibiotic resistance akin to

378 that of their monomicrobial culture counterparts, and potentially with increased
379 resistance to ciprofloxacin and trimethoprim which common front line antibiotics
380 used for UTI treatment. Due to the complexities involved in the diagnosis and
381 treatment in these infections many patients may receive inadequate antibiotic
382 treatment or indeed a lack of treatment altogether. More worryingly for patient
383 health, the majority of organisms isolated from polymicrobial cultures also
384 exhibited increased human pathogenic potential as evidenced by *in vitro* cell
385 infection assays. The diagnostic standard procedure for UTI should be
386 reconsidered in light of the data presented here.

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394

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Antibiotic concentration (µg/ml)	<i>E. coli</i> (%)		<i>E. faecalis</i> (%)		<i>P. mirabilis</i> (%)		<i>S. aureus</i> (%)		<i>P. aeruginosa</i> (%)	
	Poly (n=129)	Mono (n=21)	Poly (n=110)	Mono (n=4)	Poly (n=56)	Mono (n=1)	Poly (n=18)	Mon o (n=4)	Poly (n=46)	Mono (n=5)
Gentamicin (2)	12.4	4.76	-	-	5.3	0.0	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	0.00	15.5	0.0	0.0	0.0	-	-	-	-
Piperacillin- Tazobactam (16)	6.2	4.76	4.5	25.0	23.2	100.0	-	-	-	-
Co-amoxiclav (32)	5.4	0.00	2.7	0.0	16.1	100.0	0.0	0.0	-	-
Trimethoprim (2)	44.2	28.57	-	-	89.0	0.0	22.2	0.0	-	-
Ciprofloxacin (4)	23.3	9.52	28.2	0.0	0.0	0.0	55.5	50.0	-	-
Cephadrine (32)	28.7	19.05	-	-	55.3	100.0	50.0	25.0	-	-
Nitrofurantoin (32)	17.1	9.52	10.9	0.0			11.0	0.0	-	-
Amoxicillin (32)	45.0	42.86	7.3	0.0	37.5	100.0	66.6	75.0	-	-
Gentamicin (10)	-	-	-	-	-	-	-	-	2.17	0.0
Piperacillin- tazobactam (85)	-	-	-	-	-	-	-	-	0.0	0.0
Ceftazidime (30)	-	-	-	-	-	-	-	-	2.17	00.0
Meropenem (10)	-	-	-	-	-	-	-	-	2.17	0.0
Ciprofloxacin (1)	-	-	-	-	-	-	-	-	4.35	00.0

Table 1. Prevalence of antibiotic resistance in UTI isolates

NOTE '-' indicates that particular species/antibiotic combination was not tested.

The term 'poly' refers to strains of polymicrobial infection origin, whilst the term

'mono' refers to strains of monomicrobial infection origin.

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

β-Lactamase carriage		Percentage of strains
Any β -lactamase	- monomicrobial culture isolates	57.1
	- polymicrobial isolates	48.4
SHV	- monomicrobial culture isolates	8.6
	- polymicrobial isolates	9.8
TEM	- monomicrobial culture isolates	45.7
	- polymicrobial isolates	36.4
CTX-M	- monomicrobial culture isolates	22.9*
	- polymicrobial isolates	9.5
OXA	- monomicrobial culture isolates	11.4
	- polymicrobial isolates	4.7
Multiple β -lactamases	- monomicrobial culture isolates	22.9 [#]
	- polymicrobial isolates	8.8

strains tested were all *E. coli*, *Pseudomonas spp*, *Proteus spp*, *Citrobacter spp*, *Enterobacter spp* and *Klebsiella spp* isolated in this study. * P=0.016, #P=0.01, χ^2 test.

Figure 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 which contain the given species.

* Indicates cultures taken from polymicrobial infections

Figure 2. BOX-PCR gel of *E. coli* strains isolated from UTI samples
Lanes marked * contain the BOX-PCR profile for *Escherichia coli* CFT073, lanes marked M contain molecular weight markers. Remaining lanes show a selection of BOX profiles obtained for *E. coli* isolates.

Figure 3. Invasive capabilities of UTI *E. coli* isolates compared to *E. coli* CFT073. CFT073 invasion is designated as 1.00. Polymicrobial sample isolates are statistically more invasive than monomicrobial culture sample isolates, $P < 0.001$, χ^2 test.