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1 **Increase in bacteraemia cases in the East Midlands region of the United**
2 **Kingdom due to multi-drug resistant *Escherichia coli* ST73: High levels of**
3 **genomic and plasmid diversity in causative isolates.**

4

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20 Running title: *E. coli* ST73 genome analysis

21 Keywords: *E. coli*; bacteraemia; genomics, ESBL

22 **Abstract**

23 **Objectives** To determine the population structure of *E. coli* ST73 isolated from
24 human bacteraemia and urinary tract infections

25 **Methods** The genomes of 22 *E. coli* ST73 isolates were sequenced using the
26 Illumina HiSeq platform. High resolution SNP typing was used to create a
27 phylogenetic tree. Comparative genomics were also performed using a
28 pangenome approach. *In silico* and S1-PFGE plasmid profiling was conducted,
29 and isolates were checked for their ability to survive exposure to human serum

30 **Results** *E. coli* ST73 isolates circulating in clinically unrelated episodes show a
31 high degree of diversity at a whole genome level, though exhibit conservation in
32 gene content, particularly in virulence associated gene carriage. The isolates also
33 contain a highly diverse plasmid pool that confers multi-drug resistance via
34 carriage of CTX-M genes. All strains are highly serum resistant and uniformly
35 carry genes shown to be essential for serum resistance.

36 **Conclusions** Our data shows that a rise in incidence of multi-drug resistant *E.*
37 *coli* ST73 clinical isolates is not due to a circulating outbreak strain as in *E. coli*
38 ST131. Rather the ST73 circulating strains are distantly related and carry a
39 diverse set of resistance plasmids. This suggests that the evolutionary events
40 behind emergence of drug resistant *E. coli* differ between lineages.

41

42 **Introduction**

43 Extra-intestinal pathogenic *Escherichia coli* (ExPEC) is the term used to describe
44 strains of *E. coli* which can asymptotically colonise the intestinal tract of
45 humans and animals, but cause disease in non-intestinal sites.¹ In humans ExPEC
46 most commonly cause urinary tract infections, which is thought to affect as many
47 as 70% of the global female population.¹ ExPEC are also capable of causing
48 bacteraemia infections, where large numbers of bacterial cells gain entry to the
49 bloodstream causing a potentially life-threatening infection. The incidence of
50 bacteraemia caused by ExPEC has been increasing rapidly in the past 10 years,
51 with ExPEC now the most common cause of bacteraemia in Europe, overtaking
52 MRSA and *Clostridium difficile* bloodstream infections.²

53 The rise in cases of ExPEC bacteraemia is mirrored by a marked increase in the
54 carriage of multi-drug resistance (MDR) plasmids in ExPEC. In particular ExPEC
55 are associated with the sustained carriage and dissemination of genes encoding
56 ESBL, and especially the CTX-M variant. In some countries as many as 50% of
57 bacteraemia ExPEC isolates are ESBL positive isolates.² Numerous
58 epidemiological studies have shown the *E. coli* ST131 clone to be the most
59 commonly isolated MDR ExPEC strain type from human clinical cases.^{3,4} ST73 is
60 another phylogroup B2 strain type that is also frequently isolated from human
61 clinical cases.⁴ Unlike ST131, which has been extensively studied and
62 characterised at a population and genomic level,⁵⁻⁷ very little is known about
63 ST73 beyond the reference ExPEC strain CFT073.⁸

64 We recently conducted a molecular epidemiological survey of bacteraemia
65 ExPEC isolates from the East Midlands area of the United Kingdom.⁹ Our study
66 found that MDR ExPEC were significantly more abundant in bacteraemia

67 samples than clinical urine samples over a concomitant time frame. Perhaps
68 more surprisingly our study also showed that ST73 prevalence had risen to
69 become the most commonly isolated MDR ExPEC strain type from bacteraemia
70 samples, and not ST131 as observed in a previous study in the same region.⁴
71 Given that the rapid increase in clinical cases of MDR *E. coli* ST131 is attributable
72 to rapid global dissemination of a successful clone,^{6,7} we sought to determine if
73 the high incidence of MDR ST73 clinical isolates from our bacteraemia study was
74 also due to the emergence of a successful dominant clone.

75

76 **Methods**

77 **Bacterial isolates.** An epidemiological investigation of bacteraemia and urinary
78 tract infection (UTI) *E. coli* isolates conducted by our group in 2013 identified an
79 increase in the number of *E. coli* ST73 clinical isolates containing the CTX-M gene
80 conferring multi-drug resistance.⁹ Twenty-two isolates were selected for
81 sequencing incorporating 10 ESBL positive blood isolates, 2 ESBL negative blood
82 isolates, 3 ESBL positive UTI isolates, and 7 ESBL negative UTI isolates (table 1).
83 These were selected to represent the diversity in ESBL phenotype in the samples
84 population.

85 **Genome sequencing and analysis.** Isolates were sequenced on the Illumina
86 HiSeq2500 platform using 2 x 250bp PE sequencing (Table 1). Genome
87 assemblies were performed using Velvet and PAGIT,¹⁰ which reordered contigs
88 based on the CFT073 reference genome.⁸ Assembled genomes were annotated
89 using Prokka.¹¹ Progressive Mauve was used to create a whole genome
90 alignment of the assembled genomes.¹² High-resolution SNP typing was
91 performed by mapping fastQ files against the reference ST73 genome CFT073

92 using SMALT ([https://www.sanger.ac.uk/resources/software/smalt/#t 2](https://www.sanger.ac.uk/resources/software/smalt/#t2)) and
93 Samtools. Resulting VCF files were filtered using vcftools¹³ to retain only SNPs
94 with a MinQ 30, MinDP 10, and MinAF 0.8. The filtered VCF files were used to
95 produce a consensus sequence for each strain relative to CFT073. The sequences
96 were aligned using Mugsy¹⁴ from which a maximum likelihood phylogeny was
97 created using RaxML implementing the GTR-Gamma model.¹⁵ All raw sequence
98 data has been deposited in the European Nucleotide Archive under project
99 accession number PRJEB9931.

100 **Pangenome analysis.** A pangenome of the 22 sequenced strains and CFT073
101 was made using Gegenees.¹⁶ To determine if there were loci associated with
102 bacteraemia in ST73, the genetic content of bacteraemia isolates was compared
103 against UTI isolates using a cut-off of 80% identity across 80% of bacteraemia
104 strains, and 80% identity across 20% of UTI strains. An identical analysis was
105 conducted for ESBL positive against ESBL negative to attempt to identify loci
106 associated with ESBL carriage. Presence of virulence-associated genes¹⁷ was
107 determined by BlastN analysis of gene sequences against the de novo assembled
108 genome of each strain.

109 **Plasmid typing.** *In silico* plasmid typing was performed using a locally installed
110 version of the PlasmidFinder database.¹⁸ Assembled genomes were compared to
111 the database using BlastN to identify plasmid types present in each genome.
112 Plasmid profiling was also performed using the S1-PFGE method.¹⁹

113

114 **Results**

115 **The observed increase in MDR *E. coli* ST73 clinical isolates is due to a highly**
116 **diverse group of strains.**

117 Sequence data for all 22 isolates was mapped against the CFT073 reference
118 genome and a high-resolution SNP phylogenetic tree was constructed (Fig 1).
119 The phylogenetic tree shows that bacteraemia and UTI isolates are intermixed
120 throughout the phylogeny, as are ESBL positive and negative isolates. Pairwise
121 SNP distance calculations between isolates showed that the minimum SNP
122 distance between any two isolates was 416 SNPs, and the maximum distance
123 6,026 SNPs (Fig S1.A).

124 **Comparative genomic analysis indicates diversity between ST73 genomes**
125 **occurs at single base pair mutation level, and in plasmid repertoire.**

126 An alignment of all the ST73 genomes using progressiveMauve indicated genetic
127 variation predominantly occurring in small contigs of the assemblies (Fig S2.A)
128 suggestive that most gene-content variation occurs in plasmids and other mobile
129 genetic elements (MGE). We created a pangenome of the ST73 genomes using
130 Gegenees (Fig S2.B) showing a core genome of 3.81Mbp, and 1201 conserved
131 CDS from a total of 10,696 CDS, consistent with analyses performed on the *E. coli*
132 species and on *E. coli* ST131.^{20,21} We performed *in silico* analysis to determine the
133 presence of the major ExPEC virulence-associated genes in our data set (Fig
134 S2.C). This shows some differences in carriage of virulence genes but a relatively
135 fixed virulence gene profile. The comparison of UTI and bacteraemia isolates for
136 virulence gene carriage also showed identical profiles between the two groups.
137 We sought to identify the presence of any loci over-represented in the UTI or
138 bacteraemia group of strains, or in the ESBL positive and ESBL negative group of
139 strains using Gegenees. This analysis failed to identify any loci associated with a
140 propensity towards bacteraemia or ESBL carriage.

141 **Highly diverse plasmid repertoire in circulating clinical *E. coli* ST73**
142 **isolates.**

143 Given the observations of our pangenome analysis we sought to determine the
144 extent of MGE diversity in our ST73 isolates, focussing primarily on plasmids.
145 Using the PlasmidFinder database we performed *in silico* plasmid typing on our
146 22 isolates (Table 1). Our analysis showed that FII, FIA and FIB plasmid types
147 were predominant. To further investigate this we performed S1-PFGE plasmid
148 profiling of every isolate. No plasmids were detected in the CTX-M negative
149 isolates, but a large number of plasmid molecules were detected in the remaining
150 isolates (Table 1). A 112Kbp plasmid was found in 6 isolates which showed the
151 most similar accessory gene content in the pangenome analysis. Superimposing
152 of the plasmid typing data on the phylogenetic tree showed that the 112Kbp
153 plasmid is present in the 6 isolates that showed the lowest amount of core
154 genome diversity (Fig 1). We compared the similarity of genomes at gene
155 content level using the fragmented all-against-all comparison in Gegenees to
156 show that the 6 strains sharing the 112kb plasmid also showed gene content
157 similarity above 95% (Fig S1.B) suggesting that the plasmid pool in these 6
158 strains is highly similar if not identical.

159 **Discussion**

160 Epidemiological studies in the East Midlands area of the UK have highlighted an
161 increase in incidence of *E. coli* ST73 MDR isolates over the past 5 years.^{4,9} In this
162 study we present the genomic analysis of 22 ST73 isolates from human clinical
163 bloodstream and UTI cases, all isolated within a 3-month period from the same
164 region of the United Kingdom. Our analysis shows levels of diversity in the
165 hundreds or thousands of SNPs between isolates. This is in stark contrast to

166 ST131, where isolates from the identical UK region over a 6 month period
167 showed diversity of under 10 SNPs between strains isolated from unrelated
168 clinical episodes, and a maximum diversity of dozens of SNPs.⁵

169 Analysis of our ST73 genomic data set identified the presence of a limited
170 number of plasmid types based on *in silico rep* typing, however both genomic
171 analysis and classical plasmid profiling show plasmid diversity in the small ST73
172 population sampled here. The presence of a 112Kbp plasmid was inferred in 6
173 isolates, which were also the 6 most closely related isolates phylogenetically and
174 at gene content level. It is tempting to speculate there may be a circulating sub-
175 clone of ST73 but such inference is hampered by our small and geographically
176 restricted sample size.

177 The small population we have sequenced limits the inferences we can make from
178 our data set. However there are several key points that our study highlights. The
179 first is that the evolution and emergence of MDR lineages of ExPEC does not have
180 a one-size-fits-all model. *E. coli* ST131 became a predominant clinical ExPEC
181 isolate by clonal expansion and rapid global dissemination of an MDR clone of
182 the wider ST131 lineage.⁷ Our data of clinically unrelated ST73 isolates shows a
183 highly diverse population of circulating ST73 strains, with a diverse plasmid pool
184 driving multi-drug resistance in this lineage. In order to gain a more
185 comprehensive understanding of the emergence and population structure of this
186 important lineage of pathogenic *E. coli* it is vitally important that larger global
187 isolate collections are analysed. Equally as important is that these collections
188 include non-human reservoir isolates. By doing this we will acquire a far greater
189 understanding of the ways in which ExPEC lineages can emerge as dominant
190 MDR clinical isolates, and move our focus beyond just *E. coli* ST131.

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198 **Transparency declaration**

199 The authors declare there are no competing interests in the research conducted
200 or in the reporting of this research.

201

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257 pathogenic, drug resistant *Escherichia coli* is marked by drastic reduction in
258 detectable recombination within the core genome. *Genome BiolEvol* 2013; **5**: 699–
259 710.

260

261 **Table 1. List of isolates and genome assembly statistics used in this study**

Isolate	PCR ESBL type	Genome size (bps)	N Contigs	N50 contig size	% mapped reads	S1-PFGE plasmid profile	<i>In silico</i> Inc typing
B10	CTX-M-15	5173276	106	108731	94.5	112Kbp	FIB(AP001918), FII, Col156
B14	Negative	5099552	158	113745	90.21	Negative	
B18	CTX-M-15	5120683	125	122417	91.93	33.5Kbp, 48.5Kbp	Non-typable
B29	CTX-M-15	5261474	168	101820	93.7	112Kbp	FIB(AP001918), FII Col156
B36	CTX-M-15	5191523	152	125321	92.26	145Kbp	FIB(pB171), FII, Col156
B40	CTX-M-15	5257611	165	103459	91.43	140Kbp	FIA, FIB(AP001918)
B72	CTX-M-15	5158804	110	134654	84.53	33.5Kbp, 82 Kbp	FII(pRSB107)
B73	CTX-M-15	5150717	156	121329	94.38	112Kbp	FIB(AP001918), FII, Col156
B84	CTX-M-15	5182704	137	134972	93.42	112Kbp	FIB(AP001918), FII, Col156
B91	CTX-M-15	5155911	197	79515	90.23	120Kbp	FIB(S), FII, Col156
B102	Negative	5075956	160	87164	93.51	Negative	
B134	OXA-1 CTX-M-15	5230535	154	116039	93.61	82Kbp	FIB(AP001918), FII, FIA
U1	Negative	5243352	151	123112	86.52	Negative	
U7	Negative	5176031	145	126228	93.16	Negative	
U21	Negative	5145668	162	113459	91.81	Negative	
U24	Negative	5120446	147	110560	89.83	Negative	
U30	Negative	5287542	160	139416	87.12	Negative	
U36	Negative	5162072	138	114804	91.04	Negative	
U42	CTX-M-15	5188710	155	106920	93.92	112Kbp	FIB(AP001918), Col156, Col8282, Col(MG828)
U48	Negative	5080928	112	113440	87.44	Negative	
U50	CTX-M-15	5256879	145	117621	94.03	48.5Kbp	FII
U76	CTX-M-15	5179037	140	133761	94.11	112Kbp	FIB(AP001918), FII, Col156

262 Isolates with the prefix B were isolated from bacteraemia cases, those with prefix U from UTI. % reads
 263 mapped equates to reads mapped against the CFT073 genome
 264 N50 is a weighted median statistic such that 50% of the entire assembly is contained in contigs or
 265 scaffolds equal to or larger than this value
 266
 267

268

269 **Figure 1.** Maximum likelihood phylogenetic tree of clinical ST73 isolates, with S1-
270 PFGE and *in silico* plasmid profiling superimposed. Plasmid sizes as determined by
271 S1-PFGE, and inc-types as determined by *in silico* analysis are indicated in the legend
272 to the right. This figure appears in colour in the online version of *JAC* and in black
273 and white in the printed version of *JAC*

274

275 **Figure S1.** (A) Pairwise distance matrix of the number of SNPs difference between
276 any two isolates on the phylogenetic tree. Numbers of SNPs are relative to those
277 obtained from mapping against the CFT073 reference genome for each isolate. (B)
278 Pairwise comparison of percentage similarity between each genome at gene content
279 level, as determined by fragmented-all-against-all comparison in Gegenees. This
280 figure appears in colour in the online version of *JAC* and in black and white in the
281 printed version of *JAC*

282

283 **Figure S2.** Comparative genomics of ST73 isolates. (A) Mauve alignment of all 22
284 isolates alongside CFT073. Co-coloured blocks indicate genome segments containing
285 syntenic genetic loci. Regions to the 3' end of the alignment indicate low levels of
286 synteny. (B) Pangenome analysis of the 22 ST73 isolates alongside CFT073. Levels of
287 nucleotide identity between genomic regions are indicated as heatmap colours.
288 Green regions indicate genomic segments with levels of identity above 80% at
289 nucleotide level, down to red regions that indicate levels of identity below 20%. (C)
290 Heatmap representation of carriage of common ExPEC virulence associated genes in
291 bacteraemia and UTI isolates of ST73. This figure appears in colour in the online
292 version of *JAC* and in black and white in the printed version of *JAC*