

1 Virulence factors among isolates of extraintestinal *Escherichia*
2 *coli* (ExPEC) from hospitals in the United Kingdom.
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10 Virulence, *Escherichia coli*, neonates, K1 capsule, microcins, fimbriae, genomic
11 islands

12 **Abstract**

13 182 genes associated with virulence were sought from the whole genome sequences of all
14 non-duplicate isolates of *Escherichia coli* received by the UK Health Security Agency's
15 Antimicrobial Resistance and HealthCare Associated Infections laboratory for typing
16 between June 2019 and March 2021 from hospitals in the United Kingdom and Republic of
17 Ireland (n=593). These were from healthcare associated investigations and were not
18 associated with diarrhoeal disease. Genes that were very common or very rare were
19 excluded from further analysis. The frequency of detection of genes was compared among
20 isolates from invasive infection, screening, urine samples and from neonates. *cnf1* (coding
21 for cytotoxic necrotising factor), *clbK* (coding for colibactin), *focCDF* (coding for F1 fimbriae),
22 *kpsM_K1*, *neuBD* (associated with the K1 capsule), *mchBC*, *mcmA* (coding for microcins),
23 *papG_alleleIII* (part of a cluster encoding P fimbriae), *pic* (protein involved in intestinal
24 colonization), *sfaE/sfaoCDE* (coding for S fimbriae), *tcpC* (encoding TLR domain containing-
25 protein C) and *vat* (encoding toxin vacuolating autotransporter) were 4 to 28 times more
26 prevalent among isolates from invasive infections than among those from carriage.
27 Representatives of sequence types (ST) 12, 73, 998 and 127 carried multiple of these
28 virulence factors, no matter whether they were from screening swabs or from blood or other
29 infection sites. Isolates carrying multiple virulence factors were more prevalent from neonatal
30 screens than those from general screens. Genes associated with the K1 capsule (*ibeA*,
31 *neuBD*, *kpsM_K1*) were particularly found in STs 1193, 10, 998, 538, 80 and 141. Nanopore
32 sequencing of 14 isolates representing 10 different STs showed that the virulence elements
33 sought were largely carried in chromosomal genomic islands, which were mosaic in nature.
34 Some 9 % of isolates carried more than six of the main virulence genes/gene sets sought,
35 highlighting the potential for significant numbers of carriage isolates to cause extraintestinal
36 infections.

37

38 **Introduction**

39 Pathogenic *E. coli* are associated with various infections in humans, the most important
40 being those causing diarrhoea, urinary tract infections, sepsis and meningitis. An extensive
41 array of virulence factors has been described in the organism [1], some of which are
42 characteristic of the disease manifestation. For those causing diarrhoea, various pathotypes
43 have been defined (enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-
44 producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli*
45 (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC), each with their

46 own specific virulence factors e.g. *stx* genes are characteristic of EHEC, production of
47 enterotoxins is characteristic of ETEC and intimin (*eae*) is associated with EPEC [2].

48 For those isolates not associated with diarrhoeal illness, the situation may not be as clear
49 cut. The patient's own intestinal flora is the reservoir for these 'extraintestinal' pathogenic *E.*
50 *coli* (ExPEC), which have evolved from commensal strains by acquisition of virulence factors
51 in a cumulative manner [3-6]. Virulence factors described are many and varied and there is
52 often overlap in the virulence gene content among different extraintestinal pathotypes.
53 However, various associations have been made [1]. Uropathogenic (UPEC) *E. coli* are
54 associated with (among others) F1C fimbriae (promoting adhesion), *tcpC* (TLR domain
55 containing-protein C associated with immune evasion), cytotoxic necrotising factor *cnf1*, α -
56 haemolysin toxins, *pic* (protein involved in intestinal colonization), *sat* (secreted auto
57 transporter toxin) and *tsh* (temperature sensitive hemagglutinin). Neonatal meningitis-
58 associated (NMEC) *E. coli* are linked with the K1 capsule, with *neuB* and *neuD* genes
59 described as specific for the K1 capsule [7], *ibe* genes (of which *ibeA* is unique to *E. coli* K1),
60 S fimbriae (encoded by the *sfaABCDEFGHIHS* gene cluster) and also, in common with UPEC,
61 *cnf1* toxin; this pathotype can also be responsible for sepsis. Accordingly, *sfaE*, *sfafoCDE*
62 (coding for S fimbriae) and *tcpC* (coding for Toll/interleukin 1 receptor (TIR)-containing
63 proteins) have been associated with sepsis [8]. The toxin vacuolating autotransporter
64 encoded by *vat* has also been significantly associated with bacteraemia [9]. The term NTEC
65 is sometimes used to refer to necrotoxic *E. coli* which express *cnf1* [10]. Other potentially
66 important virulence factors include the colibactin biosynthetic cluster in the *pks* pathogenicity
67 island which is often found in combination with *cnf1*. Colibactin represents a class of
68 bacterial genotoxin inducing DNA damage and genomic instability in mammalian cells
69 [11,12]. An analysis of the prevalence of the colibactin island revealed that the *pks* island
70 was consistently associated with the yersiniabactin gene cluster [12,13]. Genotoxic *E. coli*
71 may use colibactin to compete for gut niche utilization [14]. Microcin is also important in
72 establishing colonization in the gut [15,16].

73 Many of these virulence factors are found as blocks of genes in integrative chromosomal
74 genomic islands referred to as pathogenicity islands (PAI) that are acquired by horizontal
75 transfer [17]. Since they often contain mobile elements derived from bacteriophages,
76 plasmids and insertion sequences, they are subject to rearrangements, insertions and
77 deletions and are therefore variable. These PAI are critical in providing elements encoding
78 functions (such as aiding colonization, immune evasion or adhesin and toxin production) that
79 are essential to the infection process.

80 In order to understand the importance or otherwise of the various virulence factors described
81 and to be able to identify isolates readily that are of particular virulence among those
82 submitted from healthcare associated investigations (excluding those relating to diarrhoea),
83 we sought 182 virulence factors from whole genome sequences from all isolates submitted
84 to our typing service for *E. coli* from UK hospitals over a period of 20 months during
85 2019/2020. These largely consisted of those isolated from screens, both for carbapenem
86 resistant organisms and from neonates, but also included clinical isolates from blood, urine,
87 CSF and other sterile sites. While acknowledging that all of the virulence factors play a role,
88 and that gut colonisation is an important step for strains causing extraintestinal infections, we
89 hypothesized that those isolates causing actual infections would be those most likely to
90 possess the full complement of characteristics required to cause those infections. Our panel
91 of isolates therefore provided a context against which to assess the various genes, allowing
92 us to identify a set characterising those of greatest virulence potential.

93 **Methods**

94 All isolates submitted for cross-infection and outbreak investigation to the UK Health Security
95 Agency's Antimicrobial Resistance and HealthCare Associated Infection (AMRHAI)
96 laboratory were subjected to whole genome sequencing on a NextSeq Illumina platform
97 following QIASymphony extraction and Nextera® XT library preparation. The bioinformatic
98 pipeline identified the sequence type (ST), predicted O:H type, resistance elements and,
99 where applicable, the 'SNP address'. The SNP address allows comparison of isolates within
100 the same clonal complex, with groupings at the 0, 5, 10, 25, 50, 100 and 250 SNP levels
101 [18]. The pipeline also sought 182 genes associated with virulence. The set examined,
102 consisting of 593 isolates, were received between June 2019 and March 2021 from 65
103 hospital trusts from the United Kingdom and Republic of Ireland. These consisted of non-
104 duplicate isolates from cerebrospinal fluid (CSF) (8), blood (53), urine (47), neonatal screens
105 (82), other screens (341) (consisting of 'CRE screens', rectal swabs, groin swabs and
106 faeces) and others (ascitic fluid (2), bronchoalveolar lavage (BAL) (4), bile (1), endotracheal
107 aspirate (2), endotracheal tube (ET) secretions (5), endotracheal tube tip (ETT) (5), drain
108 fluid (3), eye (5), fluid (1) foot plantar (1), line tip (1), lung swab (1), lymph node (1),
109 nasopharyngeal aspirate (1), neck (1), pancreatic abscess fluid (1), perinephric collection
110 (1), peritoneal fluid (1), sputum (10) thigh tissue (1), throat (1), wound (5) and unknown (3),
111 and the hospital environment (maserator (2), cotspace (1), sinkhole (1)). Isolates consisted
112 of 143 different sequence types, of which STs 131, 38, 69, 648, 167, 3168 and 73 were the
113 most common (120, 38, 33, 20, 17, 17 and 14 isolates, respectively). Although the number of
114 isolates from CSF were small (8), they were diverse, with each representing a different

115 sequence type. Phylogenetic groups were identified based on the combinations of the *chuA*,
116 TSPE4C2 and *yjaA* genes [19].

117 14 isolates were also subjected to nanopore sequencing using a minION Mk1C following
118 DNA extraction with a GeneJet genomic DNA kit (ThermoFisher) and library preparation
119 using the Rapid Barcoding kit SQK-RBK004 (Oxford Nanopore Technologies). Libraries
120 were pooled and concentrated using Agencourt AMPure XP beads (Beckman Coulter).
121 Sequencing was carried out on up to 10 isolates at a time on a FLO-MIN 106D Spot-On
122 flowcell (R9 version) in 72 h runs as recommended by the manufacturer. Virulence genes
123 were further sought using VirulenceFinder 2.0 on the Center for Genomic Epidemiology
124 website (<https://cge.cbs.dtu.dk/services/VirulenceFinder/>) [20]. BLAST comparisons on a
125 local database were carried out to generate Figure 3
126 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were deposited in the sequence Read
127 Archive under project [PRJNA870093](#).

128 **Results**

129 **Highly common genes**

130 Some of the genes were found in all (*aer* (a signal transducer for aerotaxis), *csgABCDEFG*
131 (encoding curli fibers), *ibeC*, *mutS*) or most (*ecpA*, *fimH*, *ibeB*, *matA*) isolates. Others that
132 were relatively common included *chuASTWXY* (associated with haem uptake), *fyuA*, *irp2*,
133 *iucABC*, *iutA* (part of the yersiniabactin and aerobactin siderophore gene clusters).

134 **Rare genes**

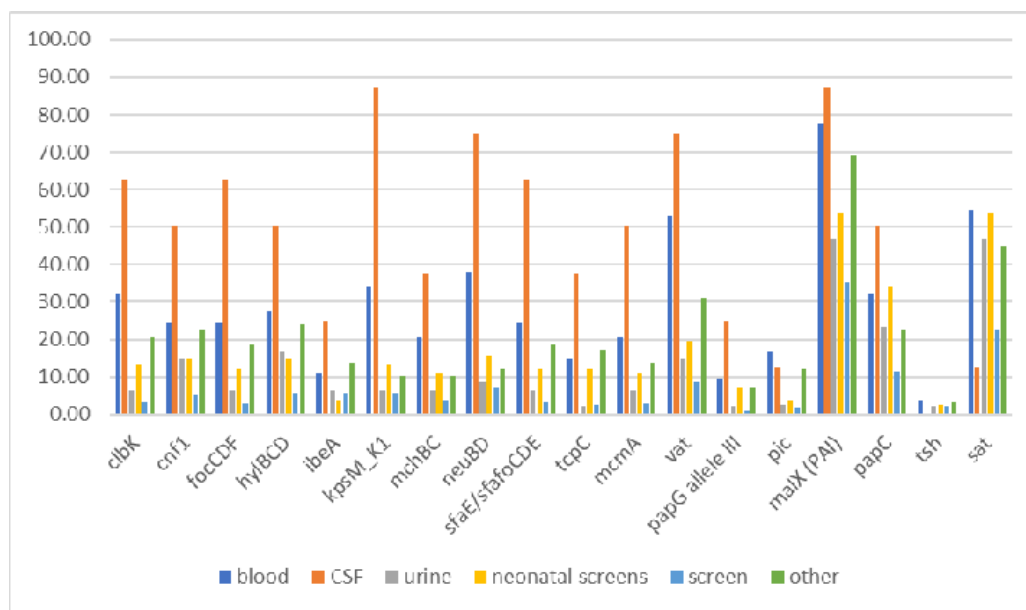
135 Other genes/gene alleles were not found in any of the isolates (*aafABCD*, *aah*, *agg3A*,
136 *agg3B*, *agg3C*, *agg3D*, *agg5A* (encoding aggregative adherence fimbriae), *aidA* (also
137 associated with adherence), *bfpA*, *bmaE*, *cdtA*, *cdtB*, *cdtC* (encoding cytolethal distending
138 toxin), *cofA*, *eae* (encoding intimin associated with ETEC), *eatA*, *espB*, *espC*, *espF*, *etpD*,
139 *f17A*, *fanA*, *fanC*, *fasA*, *fedA*, *fedF*, *fim41A*, *hylE*, *ingA*, *ipaH*, *iucD*, *K88ab*, *itcA*, *neuC*, *nleA*,
140 *papG_allele*, *papG_alleleprime*, *perA*, *pet*, *rfc*, *rpeA*, *saa*, *sta1*, *sta2*, *stb*, *subA*, *tir*). Some
141 others were only found in a very small number of isolates (10 or less) (e.g. *aaiC*, *cif*, *epeA*,
142 *espl*, *espJ*, *iha*, *ipaD*, *ingA*, *katP*, *nleB*, *nleC*, *sepA*, *toxB*, *virF*).

143 **Main study**

144 As a result of these preliminary observations, the occurrence of the following virulence
145 genes was studied in more detail: *clbK* (part of the colibactin biosynthetic cluster), *cnf1*
146 (encoding cytotoxic necrotising factor 1), *focCDF* (encoding F1 fimbriae), *hylBCD* (encoding
147 α -haemolysin toxins), *ibeA*, *neuBD*, *kpsM_K1* (associated with the K1 capsule), *mchBC*,
148 *mcmA* (encoding microcins), *safE/sfafoCDE* (encoding S fimbriae), *tcpC* (encoding TLR

149 domain containing-protein C), *vat* (encoding vacuolating autotransporter toxin), *papG*-
150 *_alleleIII*, *papC* (encoding pyelonephritis associated pili), *pic* (encoding protein involved in
151 colonization), *tsh* (encoding temperature sensitive hemagglutinin), *sat* (encoding secreted
152 autotransporter toxin) and *malX* (associated with a PAI). The percentage of isolates from
153 various isolation sites carrying these genes is shown in Figure 1 and the distribution of
154 isolates carrying from zero to 8 or more of these virulence factors (where gene clusters are
155 counted as one) is shown in Figure 2. The presence of the rarer *upaH* and *virF* virulence
156 genes was also noted.

157 Figure 1. Percentage of isolates from various isolation sites carrying each of 18 virulence
158 genes/gene sets.



159

160 **K1 capsule identified by *neuBD* and/or *KpsM_K1***

161 The K1 capsule is a PAI encoded protectin that protects the organism from phagocytosis, is
162 associated with bacteraemia and is required for crossing of the blood-brain barrier [17,21].

163 74/593 isolates were positive for *neuB* (and *neuD*); all but 10 of these were also positive for
164 *KpsM_K1* indicating that they belong to capsular type K1 associated with neonatal
165 meningitis. 20 (27 %) were from blood, 6 from CSF, 4 from urine, 3 from endotracheal
166 secretions/tube tip, 1 from a diabetic foot lesion, 1 from eye, 1 from sputum and 1 from
167 tissue; the remainder (37) were from screening swabs; at least 24 isolates were from
168 neonates. 10 % of neonatal screens were positive for these elements, while only 7 % of
169 other screens were positive for them. 38 % of blood isolates and 75 % of CSF isolates were
170 positive, suggesting a strong association with sepsis/meningitis. Isolates consisted of 21
171 different sequence types (STs) of which ST 1193 (23 isolates) and 10 (5 isolates) were the

172 most common, although some of the former were associated with clonal spread among
173 patients related in space and time. No isolates in the entire set were positive for *neuC*.

174 *Ibe* genes.

175 The *ibe* genes are so called because they are associated with invasion of brain endothelial
176 cells. *ibeA* is described as unique to *E. coli* K1, but only some isolates carried both *ibeA* and
177 *neuBD/KpsM_K1* (19). Every isolate in the entire set (593 isolates) carried *ibeC*; all but one
178 carried *ibeB*. *ibeA* was detected in 39 isolates in total, of which 5 (14 %) were from blood, 2
179 (5 %) from CSF, 3 from urine, 4 from respiratory samples, 1 from wound and 1 from eye; the
180 remainder (22) were from screening swabs. At least 10 positive isolates were from neonates,
181 but the gene was not more common among isolates from neonatal screens than from
182 screens generally (4 and 6 % respectively). The gene was found in 11 %, 25 % and 6 %
183 respectively of blood, CSF and urine isolates, compared with an overall prevalence of 7 %.
184 The 39 isolates consisted of 22 STs, of which 538 and 998 were the most common, but both
185 were associated with clonal spread among patients linked in space and time.

186 **S fimbriae (adhesin) genes**

187 Associated with NMEC and UPEC, these are the *sfa* (e.g. *safE*) and *sfafoCDE* genes. The
188 presence of *sfaE* was always accompanied with that of *sfafoCDE*. In total, 54/593 isolates
189 were positive for these genes, of which 5 were from CSF, 13 were from blood, 3 from urine,
190 1 from tissue, 1 from a line tip, 2 from eye swabs and 7 from other clinical sites; the
191 remainder were from screens (21 isolates) and the environment (1). These genes were
192 found in 25 %, 63 % and 6 % respectively of blood, CSF and urine isolates, compared with a
193 prevalence in general screens of 3 %.

194 **F1C fimbriae (adhesin) genes**

195 These are associated with UPEC and were found in 53/593 isolates in our panel. We found
196 *focCDF* to be more consistently found than *focG* and *focH*; *focC*, *focD* and *focF* were always
197 found together. Isolates carrying *focCDF* consisted of 5 from CSF, 13 from blood, 3 from
198 urine, 11 from other clinical sites, 10 from neonatal screens, 10 from general screens and 1
199 from the environment. Prevalence among blood, CSF, urine, neonatal and general screens
200 was 25, 63, 6, 12 and 3 %, respectively, with an overall prevalence of 9 %.

201 **P fimbriae**

202 P fimbriae are encoded by the *pap* operon (pyelonephritis associated pili) and mediate
203 Gal(α 1-4)Gal-specific binding via the adhesin molecule PapG. There are three major alleles
204 of *papG* (GI to GIII) of which alleles II and III are the most common in isolates causing
205 infections in humans [17,23]. We sought *papG*_allele III in particular since we had noted an
206 association with invasive isolates. We detected *papG*_alleleIII in 22/593 isolates, 6 from

207 blood, 2 from CSF, 1 from thigh tissue, 2 from ET tip, 1 from pus, 1 from urine and the
208 remainder from screens (6 from neonatal screens and 3 from adult rectal screens). The
209 isolates belonged to 12 STs, of which ST12 (9 isolates) was the most common. This allele
210 was found in 9 %, 25 %, 2 %, 7 % and 1 % of isolates from blood, CSF, urine, neonatal
211 screens, and general screens, respectively, compared with an overall prevalence of 3.5 %.

212 We detected *papC* in 110 isolates (19 %), of which 50 belonged to ST131, 10 to ST69 and 9 to ST12;
213 17 were from blood, 4 from CSF (representing 50 % of isolates from CSF), 36 from general screens,
214 28 from neonatal screens, 11 from urine, 13 from other clinical sites sites and 1 from the environment.

215 **Cnf-1 (cytotoxic necrotising factor 1)**

216 This exotoxin is associated with NMEC, UTEC and NTEC. It was detected in 68/593
217 isolates, always, in our experience, with *hyBCD* (encoding α -hemolysin), of which 4 were
218 from CSF, 7 from urine, 13 from blood and 13 from other clinical sites; the remainder were
219 from screens (30) and the environment (1). It was found in 25 %, 50 % and 15 % of blood,
220 CSF and urine isolates respectively compared with an overall prevalence of 11 %. It was
221 detected in 19 STs of which ST131 (16 isolates) and ST73 (12 isolates) were the most
222 common.

223 ***hyBCD* encoding α -haemolysin**

224 The *hyBCD* gene cluster encodes an exotoxin and is associated with UPEC, including those
225 causing upper *UTIs* such as pyelonephritis; it has been shown to target macrophages [22].
226 In our study, *hyBCD* were generally found together, with two exceptions only. They were
227 found in 74/593 isolates, of which 8 were from urine, 4 from CSF, 14 from blood and 14 from
228 further clinical sites; the remainder were from neonatal (13), other (20) and environmental (1)
229 screens. The most common ST in which the *hyBCD* cluster was found was ST131 (17
230 isolates), followed by ST73 (12 isolates).

231 **TcpC (immune evasion)**

232 *This gene encodes an inhibitor homolog of Toll-like receptors (TLR), dampening the*
233 *proinflammatory response and therefore contributing to immune evasion. It has been*
234 *associated with both sepsis and UPEC [8].* We detected this in only 41 (out of 593) isolates,
235 only 1 of which was from urine. The isolates were from CSF (3), blood (8), urine (1), other
236 clinical sites (10) and screens (9 from general screens, 10 from neonatal screens). *tcpC*
237 positive isolates accounted for 15, 38, 2, 12 and 3 % respectively of blood, CSF, urine,
238 neonatal screen and general screen isolates, with a general prevalence of 7 %.

239 **Colibactin**

240 We sought *clbK*, which is part of the colibactin biosynthetic cluster in the *pks* pathogenicity
241 island. Colibactin is a bacterial genotoxin and is consistently associated with the
242 yersiniabactin (siderophore) gene cluster. It has been associated with persistence in the gut
243 [14]. 60/593 isolates were positive for *clbK*. These consisted of 5 isolates from CSF, 17 from
244 blood, 3 from urine, 1 from tissue, 11 from other clinical sites, 11 from neonatal screens, 11
245 from general screens and 1 from the environment. These represented 32, 65, 6, 13 and 3 %
246 respectively of isolates from blood, CSF, urine, neonatal screens and other screens,
247 respectively with an overall prevalence of 10 %. Isolates belonged to 19 distinct sequence
248 types, of which STs 73, 12, 1193, 127 and 998 were the most common, in that order.
249 Despite being the most common sequence type (120/593), no representative of ST131
250 carried *clbK*. *clbK* was detected in all representatives of STs 127 and 998 and in all but one
251 representative of STs 73 and 12.

252 **Microcins**

253 Siderophore-microcins are antimicrobial peptides that are post-translationally linked to a
254 siderophore which can then kill related bacteria by mimicking iron–siderophore complexes.
255 They therefore provide a competitive advantage in colonization of the gut. They are
256 chromosomally encoded in small genomic islands that, in UPEC, interact with proteins from
257 other genomic islands carrying virulence factors to produce the active siderophore-microcin
258 [16]. We found *mchBC* in only 46 isolates of the set, consisting of isolates from CSF (3),
259 blood (11), line tip (1), pus (1), nasopharyngeal aspirate (1), ET tip (1), sputum (2), urine (3)
260 and screening isolates (9 from neonates, 13 general, 1 environmental) belonging to 22 STs
261 of which ST 73 (12 isolates) and ST12 (8 isolates) were the most common.

262 *mcmA* (microcin precursors)

263 Similarly, 46/593 isolates were positive for *mcmA*, and again isolates from CSF (4 isolates),
264 blood (11) and urine (3) were over-represented compared with their overall proportions in the
265 set. Isolates were from 20 different STs, of which ST73 (11 isolates) and ST 12 (8 isolates)
266 were the most common.

267

268 **Autotransporters**

269 Vacuolating autotransporter toxin (encoded by *vat*)

270 *vat* was detected in 103 (17 %) of the isolates, 27 from blood, 6 from CSF, 7 from urine, 18
271 from other clinical sites (ascitic fluid, thigh tissue abscess, pancreatic abscess, ET
272 secretions, line tip, ETT tip, nasopharyngeal aspirate, sputum, eye, pus), 15 from neonatal
273 screens, 29 from other screens and 1 from the hospital environment (mascerator). These
274 were over-represented in the blood, CSF and other clinical sites groups, representing 53, 75,

275 and 29 % of the isolates in each group, compared with an overall prevalence of 17 % and a
276 prevalence in screens (not neonatal) of only 9 %.

277

278 Temperature sensitive hemagglutinin (encoded by *tsh*)

279 The *tsh* gene was found in only 2 % of isolates in our panel. It was not found in any of those
280 from CSF, nor was it found in enhanced numbers in isolates from urine compared to the
281 overall prevalence or from that in screens. It was found in 3.8 % of blood isolates.

282

283 Secreted autotransporter toxin (encoded by *sat*)

284 *Sat* was detected in a third (34 %) of the isolates, with most representatives of ST131 (the
285 most common ST by far) carrying the gene. It was found in 55 %, 13 %, 47 %, 54 % and 23
286 % of blood, CSF, urine, neonatal screens and general screens, respectively.

287

288 *Pic* (protein involved in colonization)

289 The *pic* gene was detected in almost 5 % of isolates overall, with an enhanced prevalence
290 among those from blood (17 %) and CSF (13 %) compared with those from urine and
291 neonatal and general screens (3 %, 4 % and 2 %, respectively).

292

293 *upaH*

294 *upaH* is a further autotransporter gene that has been associated with biofilm formation and
295 bladder colonization [24]. Among our panel of isolates, we only detected it in 18 isolates, all
296 but one of which belonged to ST73 or a single locus variant of ST73. This ST generally is
297 associated with virulence; not surprisingly 7 of the 18 isolates were from blood and 2 from
298 line-tips, while only 5 were from screens, despite the latter greatly outnumbering those from
299 blood (53 blood isolates compared with 423 isolates from screening). The isolates were from
300 10 different hospitals, but 4 of them were clonally related to each other (5-SNP cluster) and
301 were related in space and time.

302

303 **PAI marker gene *malX***

304 *malX* codes for a phosphotransferase system enzyme that recognizes maltose and glucose.
305 It is a PAI marker. It was detected in almost all representatives of ST131 (117/ 120 isolates)
306 and was common among the panel, being found in 277/593 isolates (47 %) overall, with
307 somewhat increased prevalence among blood (41/53) and CSF isolates (7/8) (77 and 89 %,
308 respectively) compared with those from urine, neonatal and general screens (47, 54 and 35
309 % respectively). As well as ST131, it was strongly associated with STs 648 (18 isolates),
310 1193 (24 isolates) and ST73 (14 isolates).

311

312 **Prevalence of virulence genes in invasive isolates compared with screens**

313 These data do not provide sufficient support that *tsh* is more associated with invasive
314 isolates compared with screen isolates. However, there is strong evidence in support of
315 *focCDF*, *sfaE/sfafoCDE*, *clbK*, *papG_alleleIII*, *pic* and *mcmA* being associated with sepsis
316 (all greater than 7 times more prevalent among blood isolates than screen isolates), *focCDF*,
317 *sfaE/sfafoCDE*, *papG_alleleIII*, *clbK* and *mcmA* associated with CSF (all greater than 17
318 times more prevalent among CSF isolates than general screens and more than 6 times more
319 prevalent among CSF isolates than overall) as well as *cnf1*, *kpsM_K1*, *mchBC*, *neuBD*, *vat*
320 and *tcpC*. *hylBCD* and *cnf1* were associated with urinary tract infections (both greater than
321 2.8 times as prevalent among urine isolates than overall). A caveat to these observations is
322 that the number of isolates from CSF were small (8/593 isolates); however, they were
323 genetically diverse, each representing a distinct ST.

324

325 **Combinations of virulence genes.**

326 While some 355 isolates of the panel of 593 carried one or more of the virulence genes (or
327 virulence gene sets) sought (*clbK*, *cnf1*, *focCDF*, *ibeA*, *hylBCD*, *mchBC*, *neuBD*, *ibeA*,
328 *sfaE/sfafoCDE*, *tcpC*, *KpsM_K1*, *malX* (PAI), *mcmA*, *papC*, *papG*, *papG_alleleIII*, *pic*, *sat*,
329 *sepA*, *tsh*, *upaH*, *vat*, *virF*), 81 carried only one (particularly *malX*, *sat*, *tsh*, *papC*, or in one
330 instance the rare gene *virF* found in only 2 isolates in our set), 86 carried only two (with the
331 most common combinations being *malX* and *sat* (62) and *papC* and *sat* (8), and 49 carried
332 only 3 (most, all of ST 131, carrying the *malX*, *papC* and *sat* combination (30)). A number of
333 isolates including 12 from invasive sites carried the combinations of *neuBD*, *KpsM_K1*,
334 *malX*, *sat*, *vat* (15 isolates, all of ST 1193), *neuBD*, *KpsM_K1*, *malX*, *papC*, *sat* (5 isolates) or
335 *ibeA*, *neuBD*, *KpsM_K1*, *malX*, *vat* (7 isolates) (with 10 further isolates carrying one of these
336 combinations and further genes), while the combination of *cnf1*, *hylBCD*, *malX*, *papC*, *sat*
337 was found in 15 isolates (all of ST131), of which 4 were from urine (with a further 6 isolates
338 carrying this combination and further genes). Some 53 isolates (8.9 %) carried more than 6
339 of these genes/gene sets, and all but one of these carried at least 8; one carried 15 (Table
340 1). Of these 53 isolates, which all belonged to phylogroup B2, 47 carried *cbiK*; this
341 represents 78 % of all the isolates carrying *cbiK*, showing that isolates carrying this gene are
342 highly likely to carry multiple virulence factors. Many (16) carried one or more elements
343 associated with the K1 capsule (*KpsM_K1*, *ibeA*, *neuBD*). All but three carried the F1
344 fimbriae and S fimbriae genes *focCDF* and *sfaE/sfafoCDE*. Sequence types 73 (13 isolates,
345 plus 2 SLVs), 12 (8 isolates), 127 (6 isolates) and 998 (6 isolates, 4 from neonates from the
346 same hospital and 3 representing the same strain) were the most common STs among those
347 carrying 8 or more virulence genes/gene sets. Worrying, eight also carried *bla*_{OXA-48-like}
348 carbapenemase genes. In common with 3 of the 4 ST998 isolates, and 4 of the ST73

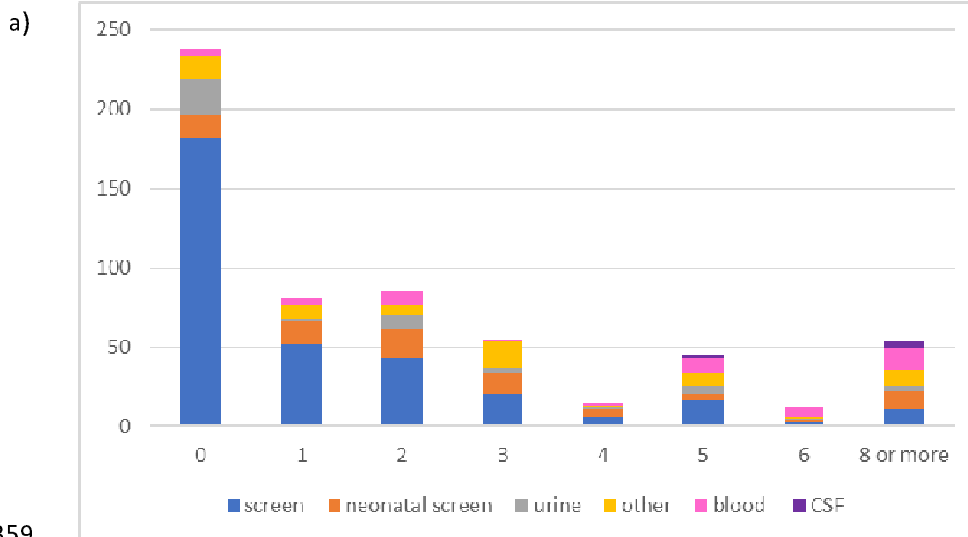
349 isolates, the two isolates belonging to ST416 and two of those belonging to ST80 were
350 epidemiologically and clonally related; all the others were not. A further two of the 13 ST73
351 isolates were from the same hospital and submitted together but were more than 250 SNPs
352 apart.

353

354 Figure 2.

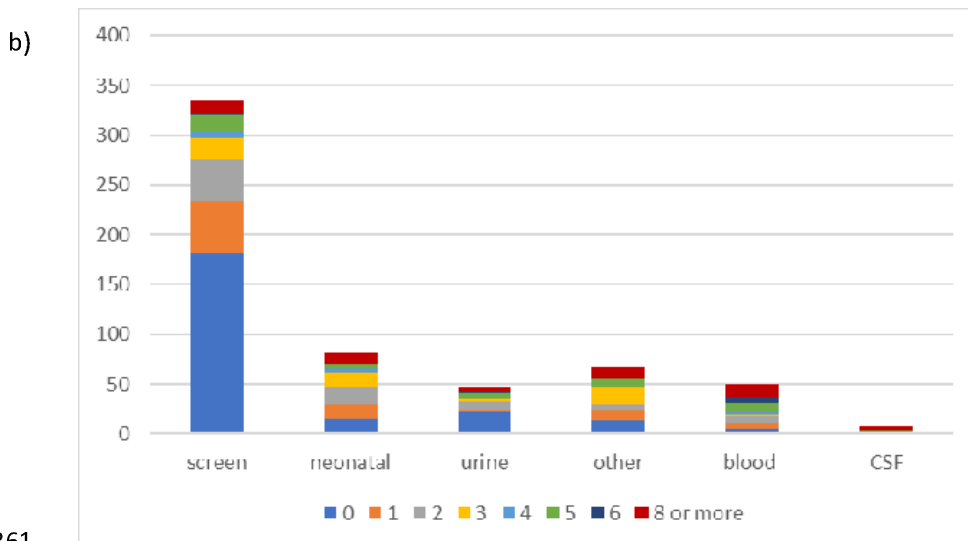
355 a) Distribution of isolates from different sites carrying 0 to 8 or more virulence
356 genes/gene sets

357 b) Distribution of number of virulence genes/gene sets among isolates from different
358 isolation sites



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363 Nanopore sequencing results

364 Nanopore sequencing was carried out on 14 isolates, consisting of 10 different sequence
365 types (STs 12, 73, 80, 127, 131, 141, 72, 416, 1859 and 4456 (Table 1). Six of the isolates
366 carried elements associated with the K1 capsule (*KpsM_K1*, *ibeA*, *neuBD*). In most cases
367 (9/14), the assemblies resulted in complete closed circular contigs for the chromosome and
368 any plasmids present; these were those included in comparisons in Figure 3. It confirmed
369 that the virulence genes sought were mostly chromosomally located in these examples. An
370 exception was the 128 kb plasmid in N6_S1_40/19, which carried the aerobactin and
371 salmochelin clusters, *iss*, *ompT*, *sitA* and *traJ* genes. In other isolates, these elements,
372 where detected, (with the exception of *traT* and *traJ*) were chromosomally located.

373 All the isolates carried *chuASTWXYU* (encoding haem uptake) in the chromosome in a gene
374 cluster that was distant from the other virulence genes sought. The three representatives of
375 ST12 from 3 different hospitals shared a similar arrangement in terms of order of genes
376 detected by the VirulenceFinder software with all sharing a section of approximately 214 kb
377 containing the genes *sitA* (coding for an iron transport protein), *iss* (increased serum
378 survival), the salmochelin cluster (e.g. *iroN* encoding a siderophore protein), *focC*, *sfaD*
379 (coding for S fimbriae/F1C minor subunit), *mcmA*, *mchF*, *mchC*, *mchB* (encoding microcins)
380 and *cea* (coding for colicin E1) (in that order). This region also included the *csgABCDEFG*
381 cluster (coding for curli fibers) and *agn43* (coding for antigen 43 involved in biofilm
382 formation). The 3 isolates shared high overall similarity with one another (with P7_NW2_18/20
383 having 99.5 % identity, 99 % coverage with N9_EE1_25/20 while P6_NW1_52/19 shared
384 99.95 % identity and 98 % coverage with it), with all carrying *terC* (encoding tellurium ion
385 resistance protein), *clbB* (coding for hybrid non-ribosomal peptide/polypeptide
386 megasynthase), *yfcV* (encoding a fimbrial protein), *gad* (coding for glutamate
387 decarboxylase), *ompT* (encoding an outer membrane protease), *vat* (encoding vacuolating
388 autotransporter toxin), *usp* (encoding uropathogenic specific protein), *fyuA*, *cnf1*, *papA*
389 (*papA_F43* or *papA_F16*), *papC*, *hra* (encoding heat-resistant agglutinin), *irp2*, *chuA*
390 (encoding outer membrane hemin receptor), *kpsE* (encoding capsule polysaccharide export
391 inner-membrane protein) with the position of *fyuA* and *irp2* (both in the yersiniabactin
392 siderophore cluster) relative to the other genes being the most variable. These isolates were
393 associated with the *kpsMIII_K96* group 3 capsule allele. The one isolate (P6_NW1_52/19)
394 carrying *bla_{OXA-48}* carried the gene in an IncL/M plasmid, as would be expected.

395 The set included 3 representatives of ST73 from 2 different hospitals, which had completely
396 different SNP addresses from one another (>250 SNP differences) showing that they were
397 not epidemiologically related. These carried *kpsMII_K5* (coding for polysialic acid transport

398 Table 1. Isolates subjected to nanopore sequencing. Each isolate was from a different patient and, with two exceptions only (hospitals NW1
 399 and WM1), from a different hospital. Isolates were labelled by patient (P1-P29, N1-N24, where those beginning with N are from neonates),
 400 hospital (by region and number within that region) and date of isolation (week/year). Regions were EE, East of England, EM, East Midlands,
 401 WM, West Midlands, L, London, NE, North East, NW, North West, SE, South East, SW, South West, S, Scotland, W, Wales, Y&H, Yorkshire
 402 and Humber.

Isolate	ST	SNP address	O:H type	Isolation site	Carbapen-emase gene	Virulence genes
P6_NW1_52/19	12	13.13.13.13.13.13	O4:H5	rectal screen	OXA-48	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, papC, papG_alleleIII, vat</i>
P7_NW2_18/20	12	1.16.16.16.16.16.17	O4:H5	blood	none detected	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, papC, papG_alleleIII, vat</i>
N9_EE1_25/20	12	1.17.17.17.17.17.18	O4:H5	faeces	none detected	<i>cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, papC, papG_alleleIII, vat</i>
P11_NE1_45/19	127	3.9.9.9.19.20.20	O6:H31	kidney urine	OXA-48	<i>clbK, cnf1, focCDF, hyI/BCD, sfaE, tcpC, malX, mcmA, papC, vat</i>
P29_WM3_39/19	131	1.4.441.470.476.510.551	O25:H4	urine	none detected	<i>cnf1, hyI/BCD, malX, papC, sat</i>
P4_EM1_41/19	141		O50O2:H6	CSF	none detected	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, neuBD, sfaE, tcpC, KpsM_K1, malX, mcmA, vat</i>
N8_L2_07/20	1859		O99:H6	blood	none detected	<i>cnf1, focCDF, hyI/BCD, ibeA, mchBC, neuBD, sfaE, KpsM_K1, malX, mcmA, pic, vat</i>
P12_NW1_38/19	372		O83:H31	CSU	OXA-48	<i>cnf1, focCDF, hyI/BCD, ibeA, mchBC, sfaE, malX, mcmA, papC, papG_alleleIII, vat</i>
N6_S1_40/19	416 (CC95)	6.27.28.28.28.29.53	O18ac:H7	blood	none detected	<i>clbK, focCDF, ibeA, neuBD, sfaE, KpsM_K1, malX, vat</i>
N10_L3_26/20	4456		O83:H4	blood culture	none detected	<i>focCDF, ibeA, mchBC, neuBD, sfaE, KpsM_K1, malX, mcmA, papC, papG, vat</i>
N5_WM1_04/20	73	56.70.70.71.72.73.74	O6:H1	line tip	none detected	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, pic, sat, upaH, vat</i>
P15_Y&H1_29/20	73	59.74.75.76.77.78.79	O6:H1	blood	none detected	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, papC, pic, sat, upaH*, vat</i>
N12_WM1_31/20	73	61.76.77.78.79.80.83	O6:H1	nasopharyngeal aspirate	none detected	<i>clbK, cnf1, focCDF, hyI/BCD, mchBC, sfaE, tcpC, malX, mcmA, pic, upaH, vat</i>
P1_SE1_24/20	80		O75:H7	urine	none detected	<i>cbiK, cnf1, focCDF, hyI/BCD, ibeA, mchBC, neuBD, sfaE, KpsM_K1, malX, mcmA, pic, vat</i>

403

404 Figure 3. Examples of BLAST comparisons of chromosomal genomic regions carrying multiple virulence genes showing mosaic nature.

405 a) region carrying *focDFI*, *sfaE*, *cnf1*, *hylABCD*, *kpsDET*, *kpsM_K1* and *aer* genes in P1_SE1_24/20 (ST80; run id RR9_BC12) compared with
406 those in P6_NW1_52/19 (ST12; run id RR10_BC07) shown in dark blue, N12_WM1_31/20 (ST73; run id RR14_BC03) shown in yellow,
407 P11_NE1_45/19 (ST127; run id RR14_BC11) shown in purple, P29_WM3_39/19 (ST131; run id RR14_BC01) shown in green,
408 P12_NW1_38/19 (ST372; run id RR9_BC02) shown in turquoise, N6_S1_40/19 (ST416; run id RR14_BC12) shown in brown, N8_L2_07/20
409 (ST1859; run id RR9_BC08) shown in pink and N10_L3_26/20 (ST4456; run id RR9_BC07) shown in grey.

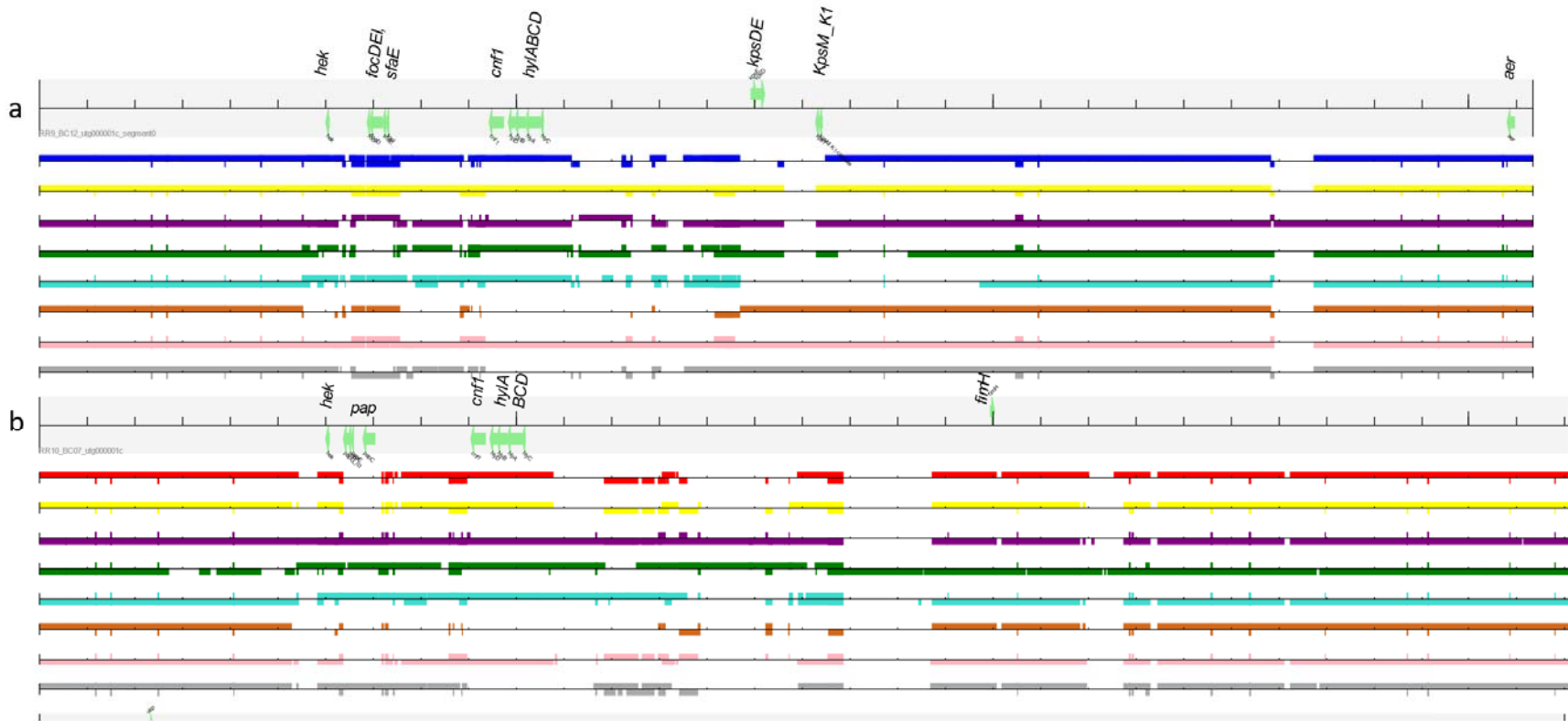
410 b) of the same region but comparison against that in P6_NW1_52/19 (ST12; run id RR10_BC07) carrying *papCEFG*, *cnf1*, *hylABCD* and *fimH*;
411 the isolate in red is P1_SE1_24/20 (ST80; run id RR9_BC12)

412 c) region carrying *usp*, *pic*, *vat* and *ecpA* in P1_SE1_24/20 (ST80; run id RR9_BC12) compared with that in the other isolates

413 d) region carrying microcin genes (*mchBCF*, *mcmA*), *csg* and salmochelin gene clusters in P1_SE1_24/20 (ST80; run id RR9_BC12) compared
414 with the other isolates

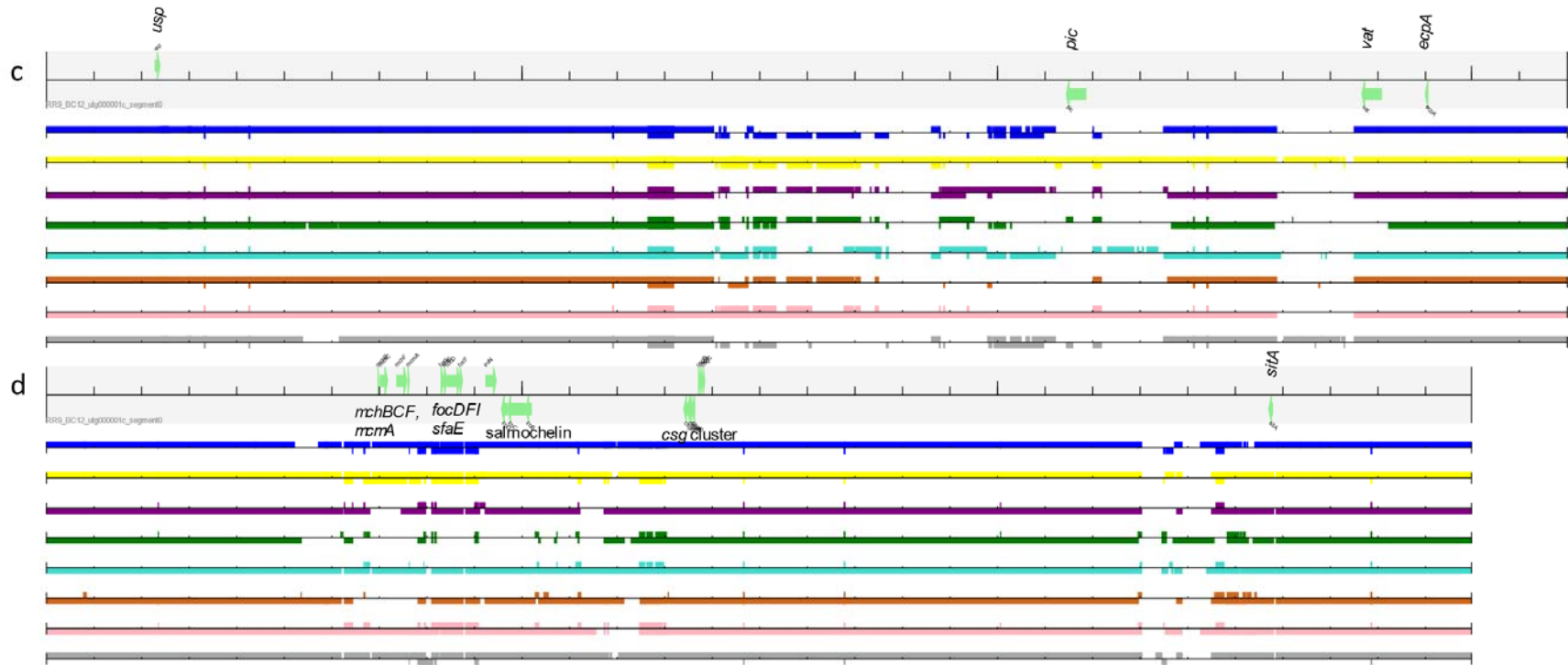
415 e) region carrying yersiniabactin and colibactin in P1_SE1_24/20 (ST80; run id RR9_BC12)

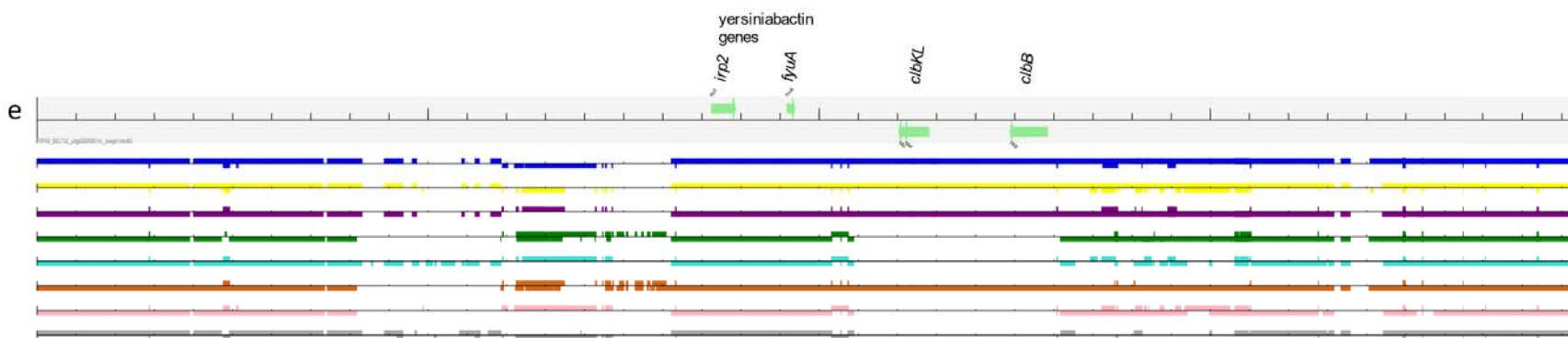
416 Note the mosaic nature of this region carrying multiple virulence genes among the isolates.



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422 protein; Group 2 capsule) but were otherwise highly similar in virulence gene content to the
423 ST12 isolates. Similarly, they carried a section carrying *sitA*, *iss*, *iroN*, *focG*, *focCsfA*,
424 *mcmA*, *mchF*, *mchC*, *mchB* genes (in that order) followed by a section carrying *ompT*, *vat*,
425 *pic* and *usp*. All also carried *chuA*, *gad*, *tcpC*, *irp2*, *fyuA*, *clbB*, *yfcV*, *terC*, *hra*, *cnf1* and *kpsE*.
426 The *cnf1* gene was in a cluster with the *hylABCD* genes.

427 Indeed, most of the isolates sequenced carried *cnf1* and *hylBCD*, which were in a
428 chromosomal genomic island (Fig. 3 a and b). Although these elements were carried by
429 most isolates, the region was nevertheless quite mosaic in nature, with some isolates also
430 carrying F1 and S fimbriae genes in this region, while others carried *pap* genes. Most of the
431 virulence genes sought (e.g. the microcin (Fig. 3d) and colibactin (Fig. 3e) genes) were
432 carried in such chromosomal islands, with clear differences in each region between isolates
433 (Fig. 3).

434 Discussion

435 While all the virulence factors are important, many were very common or very rare among
436 our set, and we have chosen to concentrate on those for which there was evidence of a
437 greater incidence among clinical isolates compared to that from screening isolates. Of
438 course, colonisation is the first step in the evolution from commensal to pathogen causing
439 extraintestinal infection, so not surprisingly, these factors were by no means confined to
440 those from infections. In agreement with previously published work, it was clear from the
441 long-read sequencing that the virulence factors were largely associated with PAIs on the
442 chromosome, which have been linked with the emergence of virulence [17]. It was also clear
443 that some sequence types, particularly STs 12, 73, 998 and 127, were strongly associated
444 with multiple virulence factors, no matter whether they were from screening swabs or from
445 blood or other infection sites. This was a consistent finding, not just within the study period,
446 but also subsequently. Alhashash et al [25] noted an increase in incidence of representatives
447 of ST73 causing clinical infections, particularly bacteraemias, and showed that, whilst they
448 were relatively diverse, they mostly shared the same complement of virulence genes, a
449 finding that was mirrored in this study. Similarly, STs 12, 80 and 998 were each associated
450 with a consistent set of virulence genes, despite coming from many different hospitals and
451 often having clearly distinct SNP addresses (at least 100 SNPs apart); STs 80 and 998 were
452 notably strongly associated with the K1 capsule. It does seem that the number of virulence
453 factors (VFs) is important, representing a continuum of acquisition in the evolution of the
454 organism from harmless commensal to one capable of causing extraintestinal infections.
455 Notably, most (99/120) representatives of ST131 carried relatively few VFs (mostly *malX* or
456 *malX*, *sat* or *malX*, *papC*, *sat* among those sought), despite being considered one of the
457 most important ExPEC lineages, highlighting the need to be aware of less well recognised
458 lineages. However, some representatives also carried *cnf1* and *hylBCD* (17), or *ibeA* (2).

459 Worryingly, almost 9 % (53/593) of isolates in our study carried more than six virulence
460 genes/gene sets, highlighting the potential for significant numbers of carriage isolates to
461 cause extraintestinal infections. Of these, 13.6 % of isolates from neonatal screens carried 8
462 or more of the VFs sought, compared with 3.3 % of other screens. While this was in part
463 associated with outbreaks in two hospitals, it is nevertheless concerning, especially
464 considering that neonatal meningitis due to *E. coli* carries a high mortality (10%) and
465 morbidity (30%) rate [26]. NMEC are characterised by K1 capsular antigens or
466 the *ibeA* invasion gene, features particularly noted in STs 1193, 10, 998, 538, 80 and 141 in
467 this study. However, these STs were not confined to isolates from neonates.

468 This work has provided a framework in which to assess isolates for virulence amongst a
469 bewildering array of candidate genes and has highlighted types that harbour the greatest
470 number of these virulence factors. It has also shown that a significant proportion of isolates
471 carry numerous virulence factors with consequent increased potential to cause infection.
472 These virulence factors are largely found in genomic islands, mosaic integrative structures
473 that can be acquired by horizontal transfer.

474

475 **Conclusions**

- 476 • *cnf1* (coding for cytotoxic necrotising factor), *clbK* (coding for colibactin), *focCDF*
477 (coding for F1 fimbriae), *kpsM_K1*, *neuBD* (associated with the K1 capsule), *mchBC*,
478 *mcmA* (coding for microcins), *papG_alleleIII* (part of a cluster encoding P fimbriae),
479 *pic* (protein involved in intestinal colonization), *sfaE/sfafoCDE* (coding for S fimbriae),
480 *tcpC* (encoding TLR domain containing-protein C) and *vat* (encoding toxin
481 vacuolating autotransporter) were more prevalent among isolates from invasive
482 infections than among those from carriage
- 483 • Representatives of STs 12, 73, 998 and 127 carried multiple of these virulence
484 factors, no matter whether they were from screening swabs or from blood or other
485 infection sites.
- 486 • Genes associated with the K1 capsule (*ibeA*, *neuBD*, *kpsM_K1*) were particularly
487 found in STs 1193, 10, 998, 538, 80 and 141
- 488 • 9 % of isolates in our study carried more than six of these virulence genes/gene sets,
489 highlighting the potential for significant numbers of carriage isolates to cause
490 extraintestinal infections
- 491 • Isolates carrying multiple virulence factors were more prevalent from neonatal
492 screens than those from general screens

- 493 • Most of the virulence factors were in chromosomal genomic islands, which are
494 mosaic in nature

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