Virulence factors among isolates of extraintestinal *Escherichia coli* (ExPEC) from hospitals in the United Kingdom.

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Virulence, *Escherichia coli*, neonates, K1 capsule, microcins, fimbriae, genomic islands

Abstract

182 genes associated with virulence were sought from the whole genome sequences of all non-duplicate isolates of *Escherichia coli* received by the UK Health Security Agency's Antimicrobial Resistance and HealthCare Associated Infections laboratory for typing between June 2019 and March 2021 from hospitals in the United Kingdom and Republic of Ireland (n=593). These were from healthcare associated investigations and were not associated with diarrhoeal disease. Genes that were very common or very rare were excluded from further analysis. The frequency of detection of genes was compared among isolates from invasive infection, screening, urine samples and from neonates. *cnf1* (coding for cytotoxic necrotising factor), *clbK* (coding for colibactin), *focCDF* (coding for F1 fimbriae), *kpsM*_K1, *neuBD* (associated with the K1 capsule), *mchBC*, *mcmA* (coding for microcins), *papG*_alleleIII (part of a cluster encoding P fimbriae), *pic* (protein involved in intestinal colonization), *sfaE/sfafoCDE* (coding for S fimbriae), *tcpC* (encoding TLR domain containing-protein C) and *vat* (encoding toxin vacuolating autotransporter) were 4 to 28 times more prevalent among isolates from invasive infections than among those from carriage. Representatives of sequence types (ST) 12, 73, 998 and 127 carried multiple of these virulence factors, no matter whether they were from screening swabs or from blood or other infection sites. Isolates carrying multiple virulence factors were more prevalent from neonatal screens than those from general screens. Genes associated with the K1 capsule (*ibeA*, *neuBD*, *kpsM*_K1) were particularly found in STs 1193, 10, 998, 538, 80 and 141. Nanopore sequencing of 14 isolates representing 10 different STs showed that the virulence elements sought were largely carried in chromosomal genomic islands, which were mosaic in nature. Some 9 % of isolates carried more than six of the main virulence genes/gene sets sought, highlighting the potential for significant numbers of carriage isolates to cause extraintestinal infections.

Introduction

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

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

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

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

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

Methods

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

115 were diverse, with each representing a different sequence type. Phylogenetic groups were
116 identified based on the combinations of the *chuA*, 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**

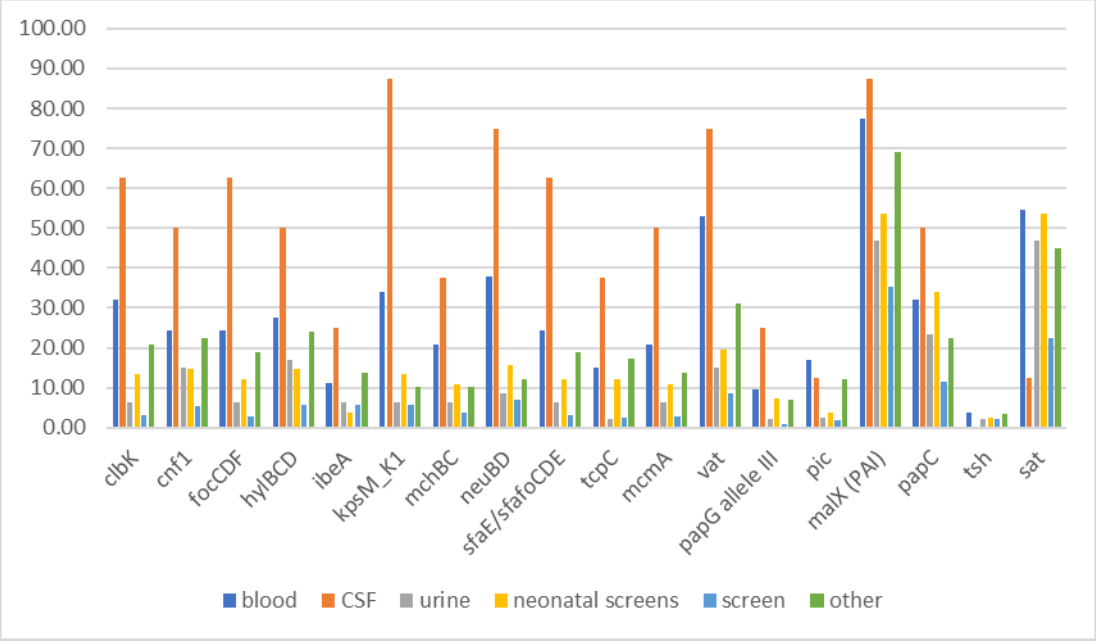
135 Other genes/gene alleles were not found in any of the isolates (*aafABCD*, *aah*, *agg3A*,
136 *agg3B*, *agg3C*, *agg3D*, *agg5A* (encoding aggregative adherence fimbriae), *aidA* (also
137 associated with adherence), *bfpA*, *bmaE*, *cdtA*, *cdtB*, *cdtC* (encoding cytolethal distending
138 toxin), *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*_allelel, *papG*_allelelprime, *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 *espI*, *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

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

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



K1 capsule identified by *neuBD* and/or KpsM_K1

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

74/593 isolates were positive for *neuB* (and *neuD*); all but 10 of these were also positive for *KpsM*_K1 indicating that they belong to capsular type K1 associated with neonatal meningitis. 20 (27 %) were from blood, 6 from CSF, 4 from urine, 3 from endotracheal secretions/tube tip, 1 from a diabetic foot lesion, 1 from eye, 1 from sputum and 1 from tissue; the remainder (37) were from screening swabs; at least 24 isolates were from neonates. 10 % of neonatal screens were positive for these elements, while only 7 % of other screens were positive for them. 38 % of blood isolates and 75 % of CSF isolates were positive, suggesting a strong association with sepsis/meningitis. Isolates consisted of 21 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(α1-4)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
213 to ST12; 17 were from blood, 4 from CSF (representing 50 % of isolates from CSF), 36 from
214 general screens, 28 from neonatal screens, 11 from urine, 13 from other clinical sites and 1
215 from the environment.

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

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

224 ***hylBCD* encoding α-haemolysin**

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

232 **TcpC (immune evasion)**

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

240 **Colibactin**

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

253 **Microcins**

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

263 mcmA (microcin precursors)

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

268

269 **Autotransporters**

270 Vacuolating autotransporter toxin (encoded by *vat*)

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

276 and 29 % of the isolates in each group, compared with an overall prevalence of 17 % and a
277 prevalence in screens (not neonatal) of only 9 %.
278
279 Temperature sensitive hemagglutinin (encoded by *tsh*)
280 The *tsh* gene was found in only 2 % of isolates in our panel. It was not found in any of those
281 from CSF, nor was it found in enhanced numbers in isolates from urine compared to the
282 overall prevalence or from that in screens. It was found in 3.8 % of blood isolates.
283
284 Secreted autotransporter toxin (encoded by *sat*)
285 *Sat* was detected in a third (34 %) of the isolates, with most representatives of ST131 (the
286 most common ST by far) carrying the gene. It was found in 55 %, 13 %, 47 %, 54 % and 23
287 % of blood, CSF, urine, neonatal screens and general screens, respectively.
288
289 Pic (protein involved in colonization)
290 The *pic* gene was detected in almost 5 % of isolates overall, with an enhanced prevalence
291 among those from blood (17 %) and CSF (13 %) compared with those from urine and
292 neonatal and general screens (3 %, 4 % and 2 %, respectively).
293
294 *upaH*
295 *upaH* is a further autotransporter gene that has been associated with biofilm formation and
296 bladder colonization [24]. Among our panel of isolates, we only detected it in 18 isolates, all
297 but one of which belonged to ST73 or a single locus variant (SLV) of ST73. This ST
298 generally is associated with virulence; not surprisingly 7 of the 18 isolates were from blood
299 and 2 from line-tips, while only 5 were from screens, despite the latter greatly outnumbering
300 those from blood (53 blood isolates compared with 423 isolates from screening). The
301 isolates were from 10 different hospitals, but 4 of them were clonally related to each other (5-
302 SNP cluster) and were related in space and time.
303
304 **PAI marker gene *malX***
305 *malX* codes for a phosphotransferase system enzyme that recognizes maltose and glucose.
306 It is a PAI marker. It was detected in almost all representatives of ST131 (117/ 120 isolates)
307 and was common among the panel, being found in 277/593 isolates (47 %) overall, with
308 somewhat increased prevalence among blood (41/53) and CSF isolates (7/8) (77 and 89 %,
309 respectively) compared with those from urine, neonatal and general screens (47, 54 and 35
310 % respectively). As well as ST131, it was strongly associated with STs 648 (18 isolates),
311 1193 (24 isolates) and ST73 (14 isolates).
312

313 **Prevalence of virulence genes in invasive isolates compared with screens**
314 These data do not provide sufficient support that *tsh* is more associated with invasive
315 isolates compared with screen isolates. However, there is strong evidence in support of
316 *focCDF*, *sfaE/sfafoCDE*, *clbK*, *papG*_alleleIII, *pic* and *mcmA* being associated with sepsis
317 (all greater than 7 times more prevalent among blood isolates than screen isolates), *focCDF*,
318 *sfaE/sfafoCDE*, *papG*_alleleIII, *cblK* and *mcmA* associated with CSF (all greater than 17
319 times more prevalent among CSF isolates than general screens and more than 6 times more
320 prevalent among CSF isolates than overall) as well as *cnf1*, *kpsM*_K1, *mchBC*, *neuBD*, *vat*
321 and *tcpC* . *hylBCD* and *cnf1* were associated with urinary tract infections (both greater than
322 2.8 times as prevalent among urine isolates than overall). A caveat to these observations is
323 that the number of isolates from CSF was small (8/593 isolates); however, they were
324 genetically diverse, each representing a distinct ST.
325
326 **Combinations of virulence genes.**
327 While some 355 isolates of the panel of 593 carried one or more of the virulence genes (or
328 virulence gene sets) sought (*clbK*, *cnf1*, *focCDF*, *ibeA*, *hylBCD*, *mchBC*, *neuBD*, *ibeA*,
329 *sfaE*/*sfafoCDE*, *tcpC*, *KpsM*_K1, *malX* (PAI), *mcmA*, *papC*, *papG*, *papG*_alleleIII, *pic*, *sat*,
330 *sepA*, *tsh*, *upaH*, *vat*, *virF*), 81 carried only one (particularly *malX*, *sat*, *tsh*, *papC*, or in one
331 instance the rare gene *virF* found in only 2 isolates in our set), 86 carried only two (with the
332 most common combinations being *malX* and *sat* (62) and *papC* and *sat* (8), and 49 carried
333 only 3 (most, all of ST 131, carrying the *malX*, *papC* and *sat* combination (30)). A number of
334 isolates including 12 from invasive sites carried the combinations of *neuBD*, *KpsM*_K1,
335 *malX*, *sat*, *vat* (15 isolates, all of ST 1193), *neuBD*, *KpsM*_K1, *malX*, *papC*, *sat* (5 isolates) or
336 *ibeA*, *neuBD*, *KpsM*_K1, *malX*, *vat* (7 isolates) (with 10 further isolates carrying one of these
337 combinations and further genes), while the combination of *cnf1*, *hylBCD*, *malX*, *papC*, *sat*
338 was found in 15 isolates (all of ST131), of which 4 were from urine (with a further 6 isolates
339 carrying this combination and further genes). Some 53 isolates (8.9 %) carried more than 6
340 of these genes/gene sets, and all but one of these carried at least 8; one carried 15 (Table
341 S1). Of these 53 isolates, which all belonged to phylogroup B2, 47 carried *cblK*; this
342 represents 78 % of all the isolates carrying *cblK*, showing that isolates carrying this gene are
343 highly likely to carry multiple virulence factors. Many (16) carried one or more elements
344 associated with the K1 capsule (*KpsM*_K1, *ibeA*, *neuBD*). All but three carried the F1
345 fimbriae and S fimbriae genes *focCDF* and *sfaE*/*sfafoCDE*. Sequence types 73 (13 isolates,
346 plus 2 SLVs), 12 (8 isolates), 127 (6 isolates) and 998 (6 isolates, 4 from neonates from the
347 same hospital and 3 representing the same strain) were the most common STs among those
348 carrying 8 or more virulence genes/gene sets. Worrying, eight also carried $bla_{\text{OXA-48-like}}$
349 carbapenemase genes. In common with 3 of the 4 ST998 isolates, and 4 of the ST73

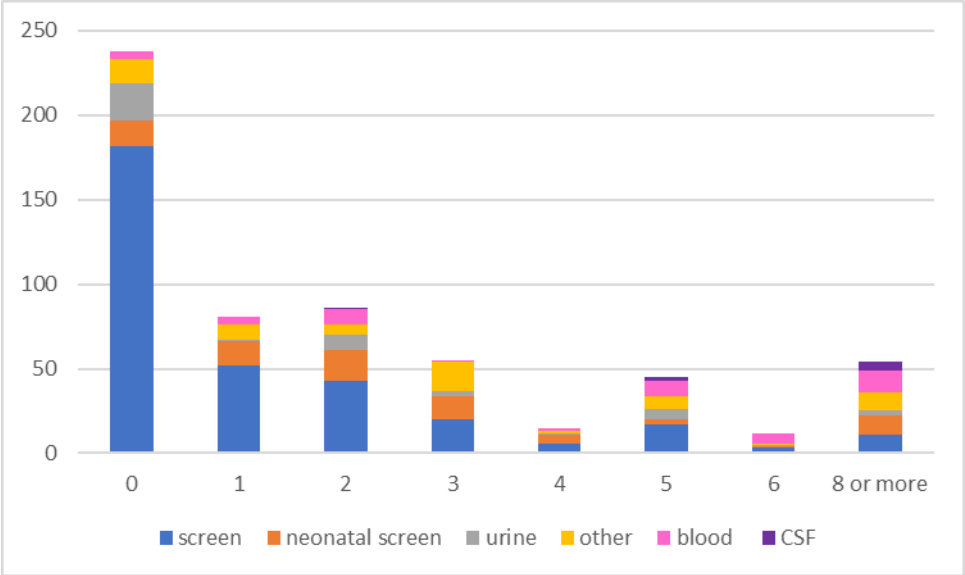
isolates, the two isolates belonging to ST416 and two of those belonging to ST80 were epidemiologically and clonally related; all the others were not. A further two of the 13 ST73 isolates were from the same hospital and submitted together but were more than 250 SNPs apart.

Figure 2.

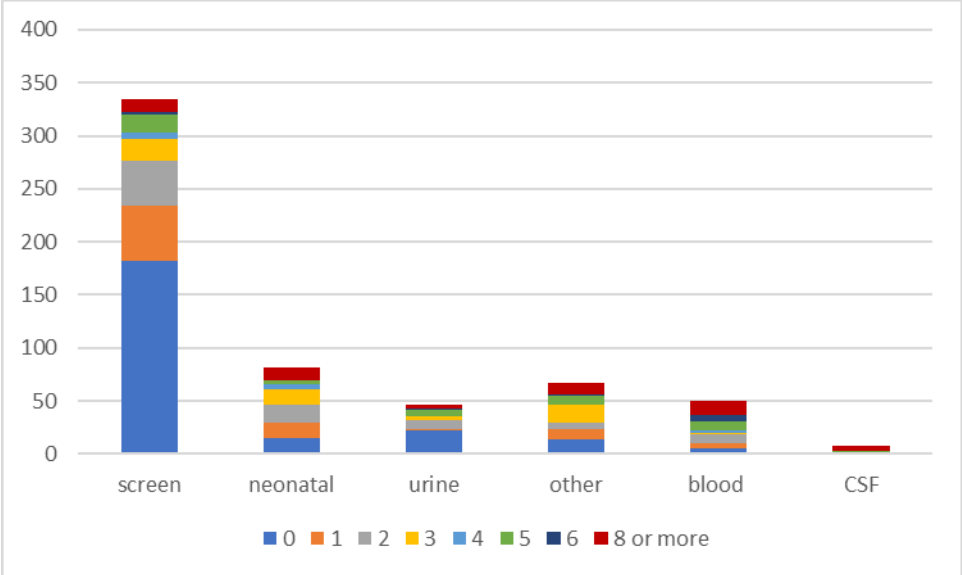
a) Distribution of isolates from different sites carrying 0 to 8 or more virulence genes/gene sets

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

a)



b)



364 **Nanopore sequencing results**

365 Nanopore sequencing was carried out on 14 isolates, consisting of 10 different sequence
366 types (STs 12, 73, 80, 127, 131, 141, 72, 416, 1859 and 4456 (Table 1). Six of the isolates
367 carried elements associated with the K1 capsule (*KpsM*_K1, *ibeA*, *neuBD*). In most cases
368 (9/14), the assemblies resulted in complete closed circular contigs for the chromosome and
369 any plasmids present; these were those included in comparisons in Figure 3. It confirmed
370 that the virulence genes sought were mostly chromosomally located in these examples. An
371 exception was the 128 kb plasmid in N6_S1_40/19, which carried the aerobactin and
372 salmochelin clusters, *iss*, *ompT*, *sitA* and *traJ* genes. In other isolates, these elements,
373 where detected, (with the exception of *traT* and *traJ*) were chromosomally located.

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

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

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

Isolate	ST	SNP address	O:H type	Isolation site	Carbapen-emase gene	Virulence genes
P6_NW1_52/19	12	13.13.13.13.13.13.13	O4:H5	rectal screen	*bla*$_{OXA-48}$	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *papC*, *papG*_alleleIII, *vat*
P7_NW2_18/20	12	1.16.16.16.16.16.17	O4:H5	blood	none detected	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *papC*, *papG*_alleleIII, *vat*
N9_EE1_25/20	12	1.17.17.17.17.17.18	O4:H5	faeces	none detected	*cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *papC*, *papG*_alleleIII, *vat*
P11_NE1_45/19	127	3.9.9.9.19.20.20	O6:H31	kidney urine	*bla*$_{OXA-48}$	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *sfaE*, *tcpC*, *malX*, *mcmA*, *papC*, *vat*
P29_WM3_39/19	131	1.4.441.470.476.510.551	O25:H4	urine	none detected	*cnf1*, *hylBCD*, *malX*, *papC*, *sat*
P4_EM1_41/19	141		O50O2:H6	CSF	none detected	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *neuBD*, *sfaE*, *tcpC*, *KpsM*_K1, *malX*, *mcmA*, *vat*
N8_L2_07/20	1859		O99:H6	blood	none detected	*cnf1*, *focCDF*, *hylBCD*, *ibeA*, *mchBC*, *neuBD*, *sfaE*, *KpsM*_K1, *malX*, *mcmA*, *pic*, *vat*
P12_NW1_38/19	372		O83:H31	CSU	*bla*$_{OXA-48}$	*cnf1*, *focCDF*, *hylBCD*, *ibeA*, *mchBC*, *sfaE*, *malX*, *mcmA*, *papC*, *papG*_alleleIII, *vat*
N6_S1_40/19	416 (CC95)	6.27.28.28.28.29.53	O18ac:H7	blood	none detected	*clbK*, *focCDF*, *ibeA*, *neuBD*, *sfaE*, *KpsM*_K1, *malX*, *vat*
N10_L3_26/20	4456		O83:H4	blood culture	none detected	*focCDF*, *ibeA*, *mchBC*, *neuBD*, *sfaE*, *KpsM*_K1, *malX*, *mcmA*, *papC*, *papG*, *vat*
N5_WM1_04/20	73	56.70.70.71.72.73.74	O6:H1	line tip	none detected	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *pic*, *sat*, *upaH*, *vat*
P15_Y&H1_29/20	73	59.74.75.76.77.78.79	O6:H1	blood	none detected	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *papC*, *pic*, *sat*, *upaH**, *vat*
N12_WM1_31/20	73	61.76.77.78.79.80.83	O6:H1	nasopharyngeal aspirate	none detected	*clbK*, *cnf1*, *focCDF*, *hylBCD*, *mchBC*, *sfaE*, *tcpC*, *malX*, *mcmA*, *pic*, *upaH*, *vat*
P1_SE1_24/20	80		O75:H7	urine	none detected	*cblK*, *cnf1*, *focCDF*, *hylBCD*, *ibeA*, *mchBC*, *neuBD*, *sfaE*, *KpsM*_K1, *malX*, *mcmA*, *pic*, *vat*

404

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

406 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
407 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,
408 P11_NE1_45/19 (ST127; run id RR14_BC11) shown in purple, P29_WM3_39/19 (ST131; run id RR14_BC01) shown in green,
409 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
410 (ST1859; run id RR9_BC08) shown in pink and N10_L3_26/20 (ST4456; run id RR9_BC07) shown in grey.

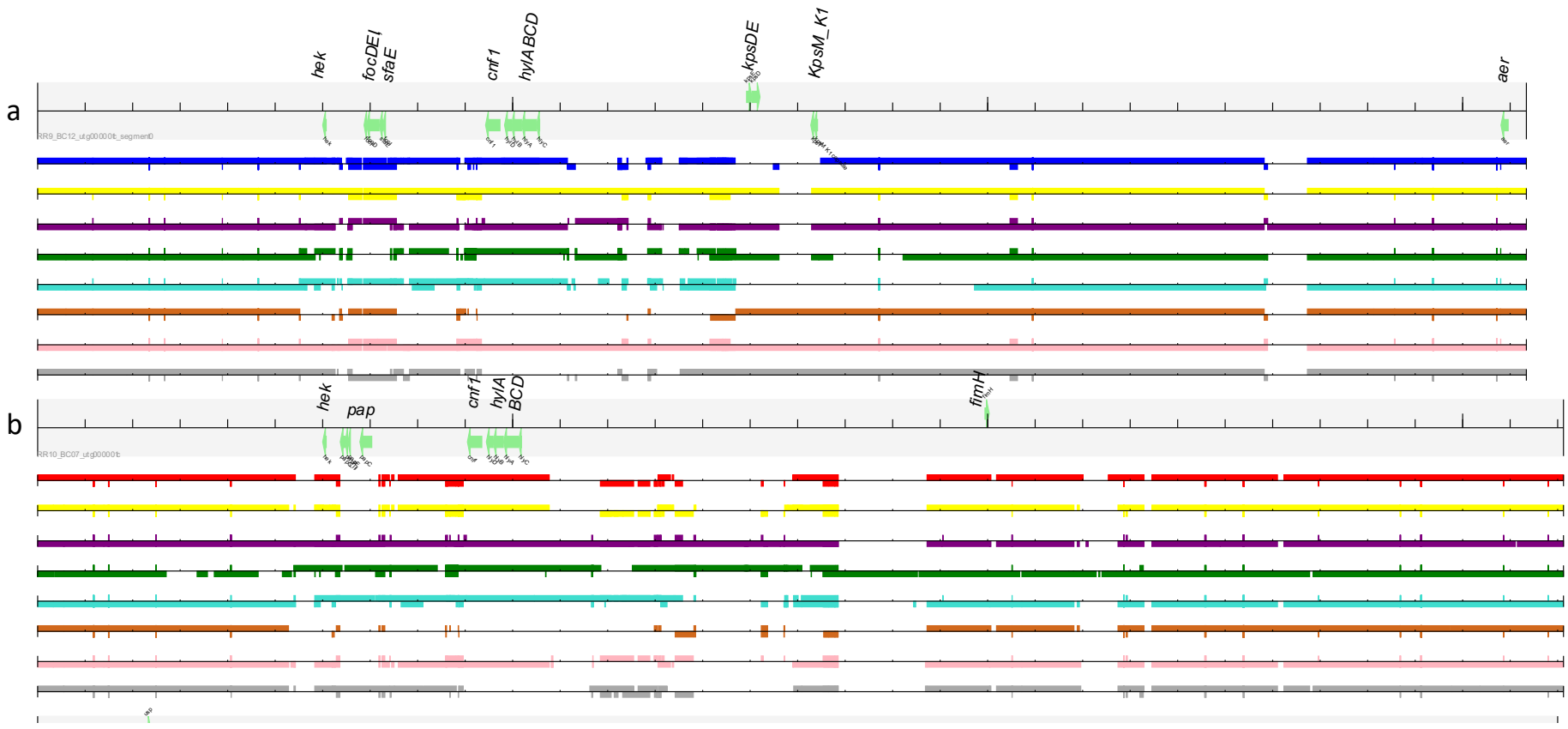
411 b) of the same region but comparison against that in P6_NW1_52/19 (ST12; run id RR10_BC07) carrying *papCEFG*, *cnf1*, *hylABCD* and *fimH*;
412 the isolate in red is P1_SE1_24/20 (ST80; run id RR9_BC12)

413 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

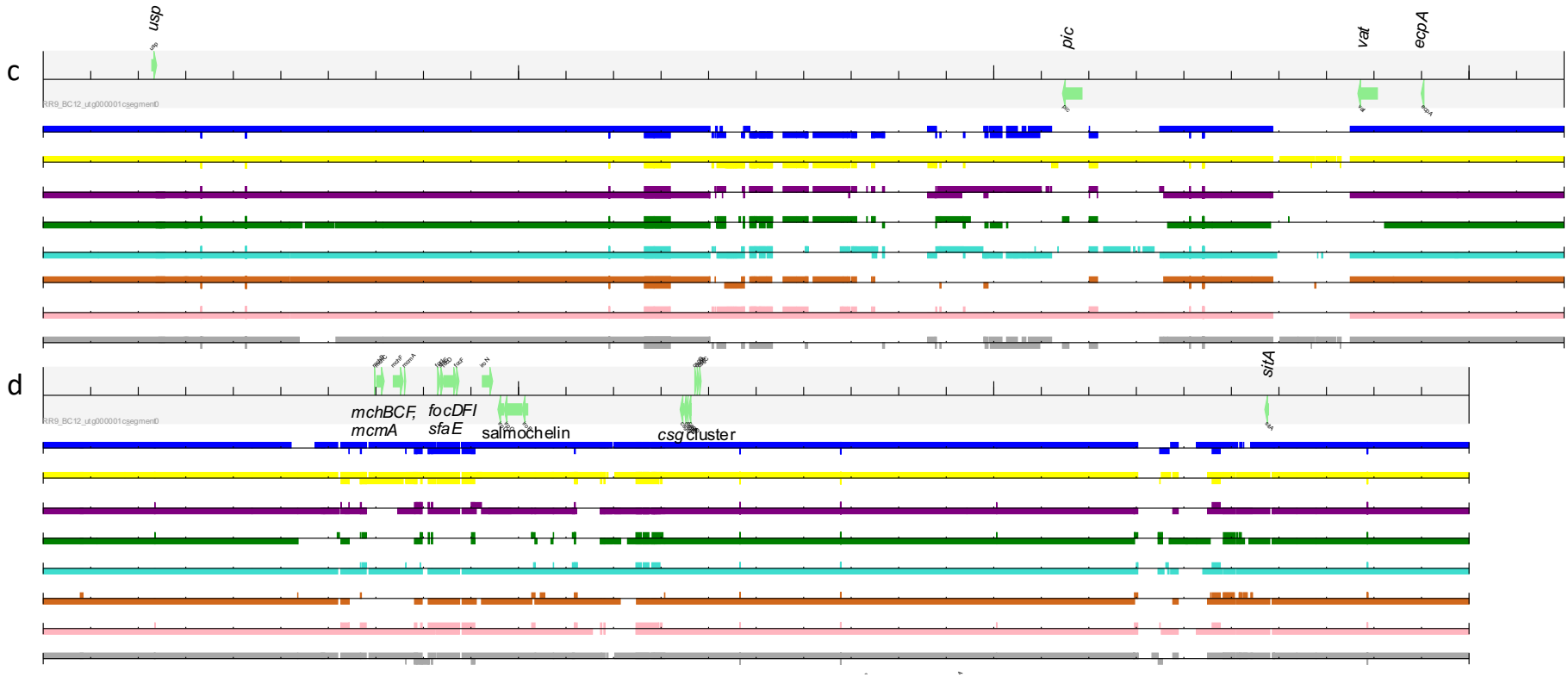
414 d) region carrying microcin genes (*mchBCF*, *mcmA*), *csg* and salmochelin gene clusters in P1_SE1_24/20 (ST80; run id RR9_BC12) compared
415 with the other isolates

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

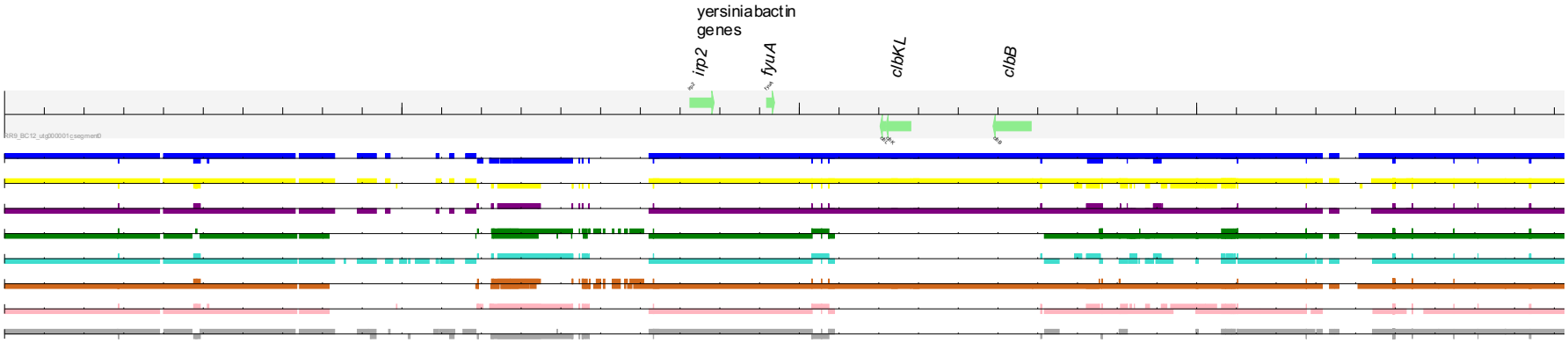
417 Note the mosaic nature of this region carrying multiple virulence genes among the isolates.


a
hek
focDEI
sfaE
cnf1
hylABCD
kpsDE
KpsM_K1
aer
b
hek
pap
cnf1
hylA
BCD
fimH

418


c
usp
pic
vat
eqpA
d
mchBCF,
mcmA
focDFI
sfaE
salmochelin
csg cluster
sitA

e



421

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*, *focCsfaE*,
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 linages. However, some representatives also carried *cnf1* and *hylBCD* (17), or *ibeA* (2).

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

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

Conclusions

- *cnf1* (coding for cytotoxic necrotising factor), *clbK* (coding for colibactin), *focCDF* (coding for F1 fimbriae), *kpsM*_K1, *neuBD* (associated with the K1 capsule), *mchBC*, *mcmA* (coding for microcins), *papG*_alleleIII (part of a cluster encoding P fimbriae), *pic* (protein involved in intestinal colonization), *sfaE/sfafoCDE* (coding for S fimbriae), *tcpC* (encoding TLR domain containing-protein C) and *vat* (encoding toxin vacuolating autotransporter) were more prevalent among isolates from invasive infections than among those from carriage
- Representatives of STs 12, 73, 998 and 127 carried multiple of these virulence factors, no matter whether they were from screening swabs or from blood or other infection sites.
- Genes associated with the K1 capsule (*ibeA*, *neuBD*, *kpsM*_K1) were particularly found in STs 1193, 10, 998, 538, 80 and 141
- 9 % of isolates in our study carried more than six of these virulence genes/gene sets, highlighting the potential for significant numbers of carriage isolates to cause extraintestinal infections
- Isolates carrying multiple virulence factors were more prevalent from neonatal screens than those from general screens

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

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