

1 **Inherent colistin resistance in Genogroups of the *Enterobacter cloacae* complex:**
2 **epidemiological, genetic and biochemical analysis from the BSAC Resistance**
3 **Surveillance Programme**

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33

34 **Abstract.**

35

36 **Background.** Polymyxins have re-entered use against problem Gram-negative
37 bacteria. Resistance rates are uncertain, with estimates confounded by selective testing.

38 **Methods.** The BSAC Resistance Surveillance Programme has routinely tested colistin
39 since 2010; we reviewed data up to 2017 for relevant Enterobacterales (n=10,914).

40 Unexpectedly frequent resistance was seen among the *Enterobacter cloacae* complex
41 isolates (n=1749); for these, we investigated relationships to species, genome, carbon
42 source utilisation and LPS structure. **Results.** Annual colistin resistance rates among *E.*

43 *cloacae* complex isolates were 4.4% to 20%, with a rising trend among bloodstream
44 organisms; in contrast, annual rates for *Escherichia coli*, *Klebsiella spp.* and *E.*
45 *aerogenes* generally remained <2%. WGS split the *E. cloacae* complex isolates into 7

46 Genogroup clusters, designated A-G. Among isolates assigned to Genogroups A-D,
47 47/50 sequenced were colistin resistant, and many, from Genogroups A-C identified as
48 *E. asburiae*. Isolates belonging to Genogroups E-G consistently identified as *E. cloacae*

49 and were rarely (only 3/45 representatives sequenced) colistin resistant. Genogroups F
50 and G – the predominant colistin-susceptible clusters – were metabolically distinct from
51 other clusters, notably regarding utilisation or not of L-fucose, formic acid, D-serine,

52 adonitol, *myo*-inositol, L-lyxose and polysorbates. LPS from resistant organisms grown
53 without colistin pressure lacked substitutions with 4-amino-arabinose or ethanolamine
54 but was more structurally complex, with more molecular species present. **Conclusions.**

55 Colistin resistance is frequent in the *E. cloacae* complex and increasing among
56 bloodstream isolates. It is associated with: (i) particular genomic and metabolic clusters,
57 (ii) identification as *E. asburiae* and (iii) with more complex LPS architectures.

58 **Introduction**

59 After long abandonment, intravenous polymyxins have re-emerged as treatments for
60 infections due to multidrug-resistant Gram-negative bacteria.^{1,2} Colistin (polymyxin E)
61 is also used as a nebulised agent for chronic pulmonary infections with *Pseudomonas*
62 *aeruginosa*,³ and as a non-absorbed oral agent in selective digestive decontamination.⁴

63 Polymyxins have multiple effects, but primarily bind to negatively-charged
64 lipid A residues in the LPS, destabilising the outer membrane and promoting their own
65 uptake.⁵ Resistance is inherent in Proteaceae, *Serratia* spp. and *Burkholderia* spp., which
66 add positively-charged sugars (4-amino-L-arabinose, Ara4N) or amino-alcohols
67 (ethanolamine) to their LPS, preventing polymyxin binding.^{1,2,5} Resistance arises in
68 other Enterobacterales via chromosomal mutations up-regulating systems that similarly
69 modify the lipid A, or through acquisition of plasmids carrying *mcr* genes, encoding
70 phosphoethanolamine transferases recruited, at least in the case of *mcr1* and *mcr2*, by
71 horizontal transfer from *Moraxella* spp.^{6,7}

72 The prevalence of colistin resistance in the UK and worldwide is uncertain and
73 subject to detection bias because many laboratories only test the drug against bacteria
74 resistant to standard agents. Moreover, mutational resistance is often unstable and
75 readily lost, with the issue muddied by ‘heteroresistance’,^{8,9} the inadequacy of disc
76 testing and the variability of MICs with the test method.¹⁰ Despite these uncertainties
77 it is clear and concerning that: (i) polymyxin-resistant mutants of carbapenemase-
78 producing Enterobacterales can cause outbreaks¹¹ and (ii) that acquired *mcr* genes are
79 widespread among *Escherichia coli* in food animals, e.g. in China.¹²

80 The BSAC Resistance Surveillance Programme¹³ has tested colistin against
81 Gram-negative bacteria (excepting inherently-resistant genera) since 2010/11. We
82 present here the results, along with an investigation of the distribution and mode of

83 resistance in *Enterobacter cloacae* complex isolates, where resistance proved to be
84 unexpectedly frequent.

85

86 **Methods and materials**

87 *Isolate collection*

88 Details of the BSAC Surveillance Programmes have been published previously.¹³
89 Briefly, these programmes collected and tested *c.* 3000 bloodstream and lower
90 respiratory tract infection (LRTI) isolates p.a. from centres across the UK and Ireland.
91 The number of centres has varied from 25 to 40, with sites asked to collect fixed quotas
92 of isolates per species group per year. There is some turnover of centres over time.
93 Collection for the Respiratory Programme runs from October to September, to capture
94 winter peaks in single years; the Bacteraemia Programme runs on the calendar year.
95 Colistin began to be tested in October 2010 for LRTI isolates and January 2011 for
96 bacteraemia isolates. Bacteraemia isolates were collected and tested by PHE's
97 Antimicrobial Resistance and Healthcare Associated Infections Reference Unit
98 (AMRHAI) throughout: LRTI isolates were collected and tested by Quotient
99 BioResearch (LGC Group, Fordam, Newmarket, UK) until the 2012/13 year and
100 thereafter by AMRHAI.

101

102 *Laboratory methods*

103 The analysis straddles a period when species identification moved from API20E[®] strips
104 (bioMerieux, Basingstoke, UK) to MALDI-ToF (Bruker, Bremen, Germany); this
105 change was introduced during the 2011-12 LRTI and 2012 Bacteraemia surveillances.
106 In general, the direct colony method was used for MALDI-ToF, though the extraction
107 method was employed for subsets of isolates (see Results). MALDI-ToF identification

108 software was updated as new versions were released - MBT DB-5627 (V.4) was
109 employed for most testing. MICs were routinely measured by BSAC agar dilution on
110 IsoSensitest Agar (Oxoid, Basingstoke, UK), with results interpreted *versus* 2019
111 EUCAST breakpoints, which score colistin as S \leq 2 mg/L, R $>$ 2 mg/L for
112 Enterobacterales. MICs were also determined by broth microdilution in Mueller Hinton
113 broth for the subset of isolates where LPS structure was studied. Heteroresistance was
114 defined as where isolated colonies ‘trailed’ in agar dilution or where tubes were
115 ‘skipped’ in broth microdilution.

116

117 *Comparison of colistin-resistant and -susceptible E. cloacae group isolates*

118 The first 50 colistin-resistant isolates collected across both surveillances in 2010-12
119 were compared, in a 1:1 case : control study, with 50 randomly-selected colistin-
120 susceptible isolates from the same centres in the same years.

121 WGS was undertaken using Illumina methodology, with phylogenetic analyses
122 based on core genomes; the read length was 2x100bp and a Nextera XT DNA library
123 Prep kit was used. Illumina pair-end sequence reads were mapped onto the genome of
124 the type strain, *E. cloacae* ATCC13047 (GenBank accession no: NC_014121.1) using
125 the Phoenix algorithm.¹⁴ SNPs were called and filtered using the Genome Analysis
126 Toolkit v2.0.¹⁵ Maximum likelihood analyses were undertaken based on the aligned
127 SNPs, allowing 20% of Ns and gaps, using RAxML under a GTRCAT model with 500
128 bootstraps.¹⁶ The best tree was drawn using the ITOL application.¹⁷ Sequence data
129 supporting these analyses are in the process of being made available in the European
130 Nucleotide Archive, under project accession number PRJEB35697.

131 The ability to metabolise carbon and energy sources was analysed using the
132 BIOLOG system (Biolog, Inc., Hayward, Ca., USA) with the PM1 MicroPlate™ test

133 panel. Bacteria were grown overnight on CLED agar, then resuspended and loaded
134 according to the manufacturer's directions. Results were recorded as growth curves by
135 the OmniLog[®] automated incubator reader then analysed using the OmniLog[®] Data
136 Collection system.

137

138 *LPS analysis*

139 Lipid A modifications were investigated using MALDI-ToF mass spectrometry (MS).
140 Bacteria were grown overnight in LB medium without antibiotic pressure, then
141 sedimented and washed thrice in 10 ml phosphate buffer (PB, 10 mM Na₂HPO₄, 1.7
142 mM KH₂PO₄). The final pellets were freeze-dried overnight then lipid A was extracted
143 as previously described.¹⁸ After de-salting, 2- μ l lipid A aliquots were loaded onto the
144 polished steel target of the MS instrument, air dried and covered by 1 μ l of 2,5-
145 dihydroxybenzoic acid matrix (Sigma Aldrich, Gillingham, UK) dissolved in 0.1 M
146 aqueous citric acid, and allowed to air dry. Finally, the target was inserted in an
147 Autoflex MALDI-ToF spectrometer (Bruker). Data acquisition and analysis were
148 performed using the Flex Analysis software.

149

150 *Statistical methods*

151 Trends in resistance prevalence were modelled by logistic regression using robust
152 standard errors to allow for clustering by year (e.g. due to experimental variation and
153 batch testing) and expressed as odds ratios; P values ≤ 0.05 were taken as moderate
154 evidence for trend. P ≤ 0.05 was also considered moderate evidence for independent
155 prediction of resistance by factors such as isolate source (blood or respiratory infection)
156 and identity. Fisher's exact test was used to assess association between WGS group and
157 substrate utilisation or colistin resistance, with Bonferroni adjustment for multiple

158 testing. Clustering of metabolic capabilities was investigated by k-means and compared
159 with hierarchical clustering by average linkage, using simple matching in both cases,
160 and with distinctness of clustering indicated by the Caliński-Harabasz stopping rule
161 (pseudo-F) index.

162

163 **Results**

164 *Distribution of colistin resistance in Enterobacterales*

165 Annual rates of colistin resistance among bloodstream and LRTI *Escherichia coli* and
166 *Klebsiella* species (including *K. aerogenes*) generally remained below 2% (Table 1).
167 Resistance was more prevalent in the *E. cloacae* complex, and further work
168 concentrated on this unexpected observation.

169 The prevalence of colistin resistance among bloodstream *E. cloacae* complex
170 isolates increased from 5.7% and 8.1% in 2011 and 2012, respectively, to 15.9% and
171 13.4% in 2016 and 2017, respectively; logistic regression indicated prevalence
172 increasing by a factor (odds ratio) of 1.19 per year (95% CI 1.07 to 1.32; p=0.001).
173 Resistance rates for LRTI *E. cloacae* complex isolates also were higher than for other
174 Enterobacterales species, but lacked the upward trend seen in bacteraemia (odds ratio
175 per year 0.91 with 95% CI 0.80 to 1.05; p=0.19); rather, resistance prevalence was
176 highest in the first year at 20% and thereafter fluctuated between 4.4% and 11.3%.
177 Logistic regression models did not support the source of the isolate (blood/respiratory)
178 as an independent predictor of colistin resistance after adjusting for species group.
179 MICs, by agar dilution, for the resistant isolates straddled between 4 and ≥ 64 mg/L,
180 compared with a sharp mode of 0.5 to 1 mg/L for susceptible isolates (Table 2).

181 Resistant isolates were collected from widely scattered sites and did not reflect
182 local outbreaks: 20/40 collecting sites submitted resistant isolates in Year 1 (i.e.

183 2010/11 for Respiratory and 2011 for Bacteraemia) 17/40 in Year 2, 15/40 in Year 3,
184 14/40 in Year 4, 17/40 in Year 5, 20/25 in Year 6 and 13/25 in Year 7.

185 According to their manufacturers, neither MALDI-TOF nor API20E® strips
186 give reliable species identification within the *E. cloacae* complex. Nevertheless, and
187 strikingly, over 85% of isolates with MICs 0.25 to 1 mg/L identified as *E. cloacae*
188 species on first test, whereas, among isolates with higher MICs (including 2 mg/L, as
189 the top border of susceptible), the proportion identified as *E. cloacae* was diminished:
190 among those with MICs >64 mg/L only 24% identified as *E. cloacae* whereas 40%
191 identified as *E. asburiae* (Table 2).

192 There were only minor differences among the source patients for the colistin-
193 resistant and -susceptible Enterobacters concerning sex (58.2% male *versus* 61.8%,
194 respectively), age distribution (≤ 4 years, 13.0% *versus* 10.3%, respectively; ≥ 60 years,
195 31.7% *versus* 30.0%, respectively) or ICU location (16.4% *versus* 11.5%). Resistance
196 to several comparator antibiotics was less prevalent among the colistin-resistant than
197 colistin-susceptible isolates, including for third-generation cephalosporins (16.4%
198 *versus* 23.2%), ciprofloxacin (6.2% *versus* 10.3%), gentamicin (2.3% *versus* 5.7%) and
199 piperacillin/tazobactam (11.9% *versus* 14.7%). Only a single (colistin-susceptible)
200 isolate was resistant to carbapenems. The proportions of isolates that were non-
201 susceptible to the various comparators was broadly stable across the surveillance period
202 reviewed.¹⁹

203

204 *Case : control comparison of colistin-resistant and -susceptible E. cloacae*

205 A detailed study of the first 50 colistin-resistant Enterobacter isolates received was
206 undertaken. These were from the 2011 and 2012 bacteraemia series and the 2010/11
207 and 2011/12 respiratory series and were compared with 50 colistin-susceptible controls,

208 randomly selected from among isolates collected at the same sites in the same period.
209 As in later periods, the resistant isolates came from multiple sites, including 33 of the
210 45 hospitals contributing isolates during the 2-years; 39 were from hospitals in England,
211 six from Ireland, and three each from Scotland and Wales. The maximum number of
212 resistant Enterobacters from any site was four (one site), with four further sites each
213 contributing three isolates. The susceptible controls were from 28 sites, with no more
214 than four from any one site.

215 WGS was performed on all 50 resistant isolates and 45/50 susceptible controls
216 and an MIC distribution for these is included in Table 2; sequencing failed for the
217 remaining 5 susceptible organisms. Analysis of the resulting data divided the 95
218 sequenced organisms into seven Genogroups, designated A-G, with a single outlier (fig.
219 1). Most (7/9) isolates in Genogroup A were colistin resistant, as were all those in
220 Genogroups B (n=9), C (n=16), and D (n=15). By contrast, resistance was seen in only
221 1/5 Genogroup E isolates, 2/26 Genogroup F isolates and 0/14 of Genogroup G. The *E.*
222 *cloacae* type strain NCTC10005/ ATCC13047 was colistin resistant (MIC 8 mg/L) and
223 fell into Genogroup D; the *E. asburiae* type strain NCTC12123/ATCC35953 fell into
224 Genogroup C but, exceptionally for this Genogroup, was colistin susceptible (MIC, 1
225 mg/L). None of the sequenced isolates carried acquired *mcr* genes.

226 Triplicate MALDI-ToF identification tests, using the extraction method and the
227 MBT DB-5627 (V.4) database, were performed on the 95 sequenced isolates.
228 Consistent identifications with all three replicates agreeing as *E. cloacae* dominated in
229 Genogroups D, E, F and G whereas members of Genogroups A, B and C were more
230 likely to identify repeatedly as *E. asburiae*, or to give a mixture of identifications as *E.*
231 *cloacae* and *E. asburiae* (Table 3).

232 Carbon source utilisation profiles were obtained for 93/100 isolates; tests for
233 the remaining 7 isolates failed. The isolates with profiles obtained included 43 of the
234 45 sequenced colistin-susceptible organisms and 44/50 sequenced colistin-resistant
235 organisms (Table 3). Further analysis, below, was based on these 87 isolates for which
236 we had both sequence and carbon source data: it excludes 6 isolates for which we had
237 carbon source data only and 7 for which we had sequence data only.

238 Strong relationships between Genogroup and metabolic profile were evident.
239 Thus, L-fucose utilisation was seen for 36/38 Genogroup F and G isolates, also 3/5 in
240 Genogroup E, whereas 42/43 isolates in Genogroups A-D tested could not use this
241 sugar. The ability to utilise glucuronamide also was largely specific to Genogroup F
242 and G, with 16/38 isolates positive *versus* 2/49 for all other Genogroups combined.
243 Adonitol utilisation was unique to Genogroup F, though only seen for 19/25 group
244 members; the ability to utilise lyxose also was largely specific to Genogroup F, with
245 12/25 isolates positive *versus* 8/62 for all other Genogroups combined. By contrast (i)
246 the ability to utilise *myo*-inositol was near-universal across Genogroups A-E, with
247 46/48 isolates positive compared with 4/38 in Genogroups F and G combined, (ii) the
248 ability to utilise formic acid was widespread in Genogroups A, B, C and E, with 34/36
249 isolates positive *versus* 2/51 for all other Genogroups combined, and (iii) the ability to
250 utilise α -keto-glutaric acid was seen in over half the Genogroup A and B isolates (5/8
251 and 6/9, respectively) *versus* 10/70 for all other groups combined. In other cases
252 *inability* to use a substrate was associated with particular Genogroups: thus 0/13
253 Genogroup G isolates utilised D-serine compared with 66/74 isolates from other
254 Genogroups and only 2/14 Genogroup C isolates utilised D-galactonic acid- γ -lactone
255 compared with 71/73 isolates from all other Genogroups combined.

256 Both the hierarchical statistical method and k-means clustering indicated that
257 Genogroups F and G (considered as a pair) were distinct from other Genogroups in their
258 substrate utilisation. Metabolic differences among the remaining Genogroups were not
259 clear-cut by statistical analyses. Since Genogroups F and G also comprised the great
260 majority of the colistin-susceptible isolates there were strong associations also between
261 metabolic profile and susceptibility, which was strongly linked (adjusted $p < 0.01$) with
262 the ability to utilise L-fucose, adonitol, and glucuronamide whereas the ability to utilise
263 formic acid and *myo*-inositol was associated with resistance ($p < 0.01$).

264 Polysorbate (Tween[®]) utilisation was more widespread among colistin-resistant
265 isolates than -susceptible isolates, with this difference more marked for polysorbate 80
266 (adjusted $p=0.004$) than polysorbate 40 (adjusted $p=0.011$) or polysorbate 20 (adjusted
267 $p = 0.439$). Underlying these observations were two key traits: (i) that the great majority
268 of Genogroup G isolates (colistin-susceptible) lacked the ability to utilise any
269 polysorbate whereas all the predominantly colistin-resistant Genogroups (A-D)
270 comprised organisms that mostly could utilise polysorbates 20 and 40 and, (ii) that
271 Genogroup D (colistin resistant) and F (mostly colistin-susceptible) isolates commonly
272 utilised polysorbates 20 and 40 but not polysorbate 80.

273

274 *LPS analysis*

275 One or two representatives of each Genogroup cluster were selected for LPS
276 characterisation along with the *E. cloacae* type strain NCTC10005/ATCC13047, which
277 was colistin resistant and belonged to Genogroup D. Broth dilution MICs were
278 determined for these isolates in addition to the initial agar dilution values. The values
279 in broth were higher, particularly for the more resistant organisms but only in one case

280 was there a categorical disagreement: isolate EN104606 was found susceptible on agar
281 (and thus atypical of its Genogroup [A]) but heteroresistant in broth (Table 4).

282 MALDI-TOF MS analysis of the lipid A of *E. cloacae*
283 NCTC10005/ATCC13047 (fig. 2), grown without antibiotic pressure, revealed
284 prominent molecular ion peaks at 1387, 1797, 1840 and 2063 m/z . The 1387 m/z peak
285 is consistent with tetra-acylated lipid A fragmentation product, while the 1824 m/z peak
286 is consistent with hexa-myristoylated (C14) lipid A forms resembling those found in *K.*
287 *pneumoniae*, *E. coli* and *Yersinia enterocolitica*.^{20,21,22} The species at 1797 m/z is
288 consistent with a penta-myristoylated lipid A substituted with an additional C12 acyl
289 chain (fig. 2). Modified forms of the 1824 and 1797 m/z molecular ions were also
290 present, reflecting addition of a hydroxyl group (typically catalysed by LpxO; m/z 1840
291 to 1824) or a palmitate group (typically catalysed by PagP) (m/z 2063 to 1824).²⁴ These
292 results indicate that the lipid A of strain ATCC13407 is complex and modified by 2-
293 hydroxylation and palmitoylation. No molecular ions consistent with Ara4N or
294 phosphoethanolamine substitutions were observed using bacteria grown in drug-free
295 LB; an analysis of lipid A modifications under other *in vitro* growth conditions and *in*
296 *vivo* will be reported elsewhere.

297 Lipid A analyses for representatives of the various DNA Genogroups are
298 summarised in Table 4. The 1824 m/z molecular ion was abundant in the lipid A of
299 most isolates except N2878 (Genogroup E) (Table 4). Most of the isolates produced
300 penta-myristoylated lipid A with an additional C12 acyl chain (1797 m/z), although the
301 abundance of this ion varied (Table 4 and fig. S1). In general, more complex patterns,
302 with a greater number of molecular species, particularly including modified forms with
303 hydroxylation and palmitoylation, were observed for isolates with colistin resistance or
304 heteroresistance (Table 4).

305

306 **Discussion**

307 Colistin began to be tested in the BSAC Surveillance in 2010 and we reviewed
308 subsequent resistance trends among Enterobacterales. Resistance prevalence remained
309 under 2% in almost all years for *E. coli* and *Klebsiella* (including *K. aerogenes*) but
310 was higher for bloodstream and respiratory *E. cloacae* complex isolates, with a strong
311 increasing trend for bloodstream, but not respiratory, organisms. The colistin-resistant
312 *E. cloacae* isolates were widely scattered in time and place and did not represent
313 outbreak clones, as confirmed by WGS. Rather, they largely belonged to Genogroups
314 A-D and had metabolic differences from members of Genogroups F and G, which,
315 along with Genogroup E, encompassed the great majority of colistin-susceptible *E.*
316 *cloacae*. Within Genogroups A-D, 47/49 isolates were colistin resistant compared with
317 3/45 isolates in Genogroups E-G.

318 Relating our Genogroups to the established taxonomy for *the E. cloacae*
319 complex proved challenging. Although MALDI-ToF does not reliably identify within
320 the *E. cloacae* complex it was striking that isolates belonging to three of the four
321 predominantly colistin-resistant Genogroups (A, B and C but not D) were more likely
322 to identify as *E. asburiae*, or to give a mixture of *E. asburiae* and *E. cloacae*
323 identifications, with this likelihood rising with the colistin MIC. Conversely, isolates
324 belonging to the predominantly susceptible Genogroups E, F and G largely identified
325 as *E. cloacae*, as did the (mostly resistant) members of Genogroup D. The simple notion
326 of intrinsic colistin resistance in *E. asburiae* and susceptibility in *E. cloacae* is however
327 refuted by the literature²⁵ and by the type strains *E. asburiae* NCTC12123/ATCC35953
328 (Group C, MIC 1 mg/L) and *E. cloacae* NCTC10005/ATCC13047 (Group D, MIC 8
329 mg/L) showing the converse pattern.

330 Grimont and Grimont write, in *Bergey's Manual of Determinative*
331 *Bacteriology*²⁶ that 'typical' *E. cloacae* largely fall into their genomic group 3. This
332 putatively corresponds to our Genogroups F and G, as evidenced (i) by the dominance
333 of these genomic groups among 'typical' colistin-susceptible isolates and (ii) by ability
334 of Genogroup F and G isolates to utilise adonitol and fucose, which are not utilised by
335 Bergey's other genomic groups - and inability to utilise *myo*-inositol- a substrate
336 Bergey notes as less reliably used by group 3 than by other groups. Our Genogroups A-
337 D predominantly comprised organisms selected on the basis of their colistin resistance
338 – a trait unlikely to be common in series selected on other criteria –so it is perhaps
339 unsurprising that they do not obviously match to other genomic groups described by
340 Grimont and Grimont. We agree that the 'type strain' *E. cloacae*
341 NCTC10005/ATCC13047 is poorly representative, as it clusters apart from typical (i.e.
342 F and G / group 3) isolates, falling into our Genogroup D and Grimont and Grimont's
343 group 1.

344 In general, colistin resistance depends on the production of modified lipid A
345 molecules with substitutions that reduce the electronegative potential of the LPS. The
346 lipid A profiles of selected isolates representing the various *E. cloacae* clusters were
347 investigated after growth without antibiotic pressure to assess if isolates displaying high
348 resistance or heteroresistance to colistin were "primed" for resistance. Compared with
349 susceptible isolates, the resistant and heteroresistant organisms examined exhibited
350 complex lipid A patterns, more often having forms with 2-hydroxylation and
351 palmitoylation, which typically are generated by LpxO and PagP enzymes. These
352 modifications are associated, in several bacteria, with enhanced resistance to
353 polymyxins and vertebrate antimicrobial peptides,²⁷⁻²⁹ and they are predicted to reduce
354 permeability across the outer membrane.²⁹ The expression of *lpxO* and *pagP* genes is

355 regulated by the master two-component system PhoPQ,^{30,31} which controls enzymes
356 involved in the remodelling of the LPS, including those that add Ara4N and
357 ethanolamine. Although expression of *lpxO* and *pagP* homologues was not investigated
358 here, our results suggest that the heteroresistant isolates have a higher basal level of
359 expression of these genes, which might confer some immediate protection against
360 colistin, with this further enhanced by full activation of the PhoPQ regulon in the
361 presence of the antibiotic. Recent work suggests that LpxO expression is also regulated
362 by the metabolic and redox status of the bacterium,³² suggesting that heteroresistance
363 could also reflect bacterial adaptation to metabolic stress.

364 The study has several limitations, besides the taxonomic uncertainty within the
365 *E. cloacae* complex. First, agar dilution was used for susceptibility testing whereas
366 broth microdilution is now recommended by EUCAST as more reliable for resistance
367 detection, though this view is disputed by others.¹⁰ At most, however, this may have
368 led us to underestimate the prevalence and degree of resistance; for the isolates
369 subjected to LPS analysis (Table 4) broth MICs were determined, in addition to the
370 original agar values. The broth values typically were higher but, only in one case did
371 this lead to a categorical disagreement: isolate EN104606 was found susceptible – and
372 so atypical of Genogroup A, to which it belonged, by agar dilution – but proved
373 heteroresistant in broth testing. A second caveat is that colistin MICs were very widely
374 distributed among resistant isolates and an organism with an MIC of 4 mg/L may have
375 more in common with a ‘susceptible’ *E. cloacae* than one with an MIC of >64 mg/L.
376 Third, this project had a long gestation and WGS and metabolic profiling were
377 exclusively done on isolates from the early years. It is plausible that proportions of the
378 different Genogroups changed subsequently and that expansion of one or more of this

379 predominantly resistant groups A-D, with particular pathogenic traits, explains the
380 dichotomy in trends for bloodstream and LRTI isolates.

381 The practical importance of our observations are uncertain. On the one hand, *E.*
382 *cloacae* is among the more frequent Gram-negative opportunists and, along with *E. coli*
383 and *K. pneumoniae*, is among the major hosts of carbapenemases, forcing colistin use.
384 Accordingly, any potential ‘loss’ of colistin, e.g. via an expansion of one or more
385 resistant Genogroup, would potentially be concerning. On the other hand, intravenous
386 polymyxins are now being supplanted by β -lactamase inhibitor combinations, including
387 ceftazidime/avibactam and meropenem/vaborbactam, which are less toxic and appear
388 more efficacious against Enterobacterales with KPC (and OXA-48 in the case of
389 ceftazidime/avibactam) carbapenemases.³³ If these and coming analogues including
390 imipenem/relebactam, cefiderocol and cefepime/zidebactam live up to their promise,
391 the renaissance of intravenous polymyxins may prove to be a brief interlude, and the
392 erosion of *Enterobacter* coverage less than catastrophic.

393

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408

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410 **DML:** Advisory Boards or ad-hoc consultancy Accelerate, Allecra, Antabio, Centauri,
411 Entasis, Integra-Holdings, Meiji, Melinta, Menarini, Mutabilis, Nordic, ParaPharm,
412 Pfizer, QPEX, Roche, Shionogi, T.A.Z., Tetraphase, VenatoRx, Wockhardt, Zambon,
413 Paid lectures – Astellas, bioMerieux, Beckman Coulter, Cardiome, Cepheid,
414 Merck/MSD, Menarini, Nordic, Pfizer and Shionogi. Relevant shareholdings or options
415 – Dechra, GSK, Merck, Perkin Elmer, Pfizer, T.A.Z, amounting to <10% of portfolio
416 value. **SM, RA, AC, BP:** nothing to declare but PHE’s AMRHAI Reference Unit has
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424 Dohme, Meiji Seika, Mobidiag, Momentum Biosciences, Neem Biotech, NIHR, Nordic
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430

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529

Table 1. Rates of resistance to colistin among Enterobacterales species collected in the BSAC Surveillance Programme

	Proportion of isolates found resistant to colistin, 2 mg/L						
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7
Bacteraemia	2011	2012	2013	2014	2015	2016	2017
<i>Enterobacter cloacae</i> group*	9/158	13/161	12/160	11/172	17/169	27/170	21/157
<i>K. aerogenes</i> (<i>E. aerogenes</i>)	0/14	0/36	0/44	0/36	1/41	1/29	1/29
<i>K. pneumoniae</i>	2/200	4/196	1/213	1/205	8/206	2/186	3/164
<i>K. oxytoca</i>	0/56	0/55	0/53	0/55	0/57	0/58	0/57
<i>E. coli</i>	2/522	3/520	6/539	3/547	3/548	1/496	2/477
LRTI	2010/11	2011/12	2012/13	2013/14	2014/15	2015/16	2016/17
<i>Enterobacter cloacae</i> group*	21/105	7/93	10/85	3/68	9/90	10/90	8/71
<i>K. aerogenes</i> (<i>E. aerogenes</i>)	0/34	0/42	0/40	0/22	0/23	0/35	0/32
<i>K. pneumoniae</i>	4/145	4/166	2/140	3/150	4/187	0/152	2/141
<i>K. oxytoca</i>	3/71	1/73	1/56	0/68	0/63	1/68	0/49
<i>E. coli</i>	6/274	1/239	0/250	0/255	0/244	0/230	3/277

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Bold text indicates rates >2%

**Enterobacter cloacae* group comprises: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis*.

Until 2014/15 (respiratory) and 2015 (bacteraemia) isolates were collected from up to 40 centres across the UK and Ireland and, thereafter from up to 25 centres.

536 **Table 2.** Species identification within the *E. cloacae* group in relation to colistin MIC: all isolates.
 537

Colistin MIC (mg/L)	Whole collection 2010-17: (%) isolates identified as:					Isolates sequenced
	Total	<i>E. cloacae</i>	<i>E. asburiae</i>	Other named	No species-level identification	
≤0.25 (S)	184 (100)	169 (91.8)	8 (4.3)	6 (3.3)	1 (0.5)	21
0.5 (S)	990 (100)	880 (88.9)	54 (5.5)	3 (0.3)	53 (5.4)	23
1 (S)	360 (100)	311 (86.4)	20 (5.6)	2 (0.6)	27 (7.5)	1
2 (S, breakpoint))	37 (100)	24 (64.9)	9 (24.3)	0 (0)	4 (10.8)	
4 (R)	26 (100)	13 (50.0)	10 (38.5)	1 (3.8)	2 (7.7)	7
8 (R)	31 (100)	21 (67.7)	6 (19.4)	0 (0)	4 (12.9)	8
16 (R)	25 (100)	17 (68.0)	2 (8.0)	3 (12.0)	3 (12.0)	12
32 (R)	21 (100)	10 (47.6)	2 (9.5)	1 (4.8)	8 (38.1)	
≥64 (R)	75 (100)	18 (24.0)	30 (40.0)	2 (2.7)	25 (33.3)	23
Total	1749 (100)	1463 (83.6)	141 (8.1)	18 (1.0)	127 (7.3)	95 ^a

538

539 ^a Omits 5 isolates for which sequencing failed

540 **Note:** From 2010 to 2012 identification was by API20E strips; subsequently identification was by MALDI-ToF using the colony method (see
541 text). Neither method gives reliable definitive identifications within the *E. cloacae* complex and ‘identifications’ were apt to vary upon repeat
542 testing; nevertheless the trend to higher proportion identified as *E. asburiae* or not identified to species level at higher colistin MIC is clear.
543

544 **Table 3.** Metabolic traits in relation to colistin status and WGS-based Genogroup

	In relation to Colistin MIC (mg/L)		In relation to Genogroup							
	≤2	>2	A	B	C	D	E	F	G	Outlier
No. group	43	44	8	9	14	12	5	25	13	1
No. able to metabolise										
Dulcitol	3	8	1	5	1	1	0	2	0	1
D-Serine	27	39	8	5	13	12	5	22	0	1
L-Fucose	37	3	0	0	0	1	3	23	13	0
Formic acid	6	30	8	9	12	2	5	0	0	0
D-Galactonic acid-γ-lactone	42	31	8	8	2	12	5	25	13	0
L-Rhamnose	43	38	8	9	8	12	5	25	13	1
D-Melibiose	36	42	7	9	13	12	5	24	8	1
α-Keto-glutaric acid	6	15	5	6	3	0	0	6	1	0
α-Methyl-D-galactoside	37	42	7	9	13	12	5	24	8	1
Lactulose	20	13	6	1	5	2	2	9	8	0
α-Hydroxy butyric acid	9	4	1	0	2	1	1	5	3	0
Adonitol	17	2	0	0	0	0	0	19	0	0

Glycyl-L-aspartic acid	40	36	8	9	13	6	3	25	12	645
<i>myo</i> -Inositol	9	42	7	9	13	12	5	4	0	1546
Mono-methyl succinic acid	4	10	3	0	6	1	0	3	1	0
L-Lyxose	16	4	0	2	1	0	1	12	3	1
Glucuronamide	16	2	1	0	1	0	0	9	7	0
Phenylethylamine	5	2	0	1	0	0	0	5	1	0
Polysorbate (Tween®) 20	23	35	7	9	11	8	5	16	2	0
Polysorbate (Tween®) 40	20	37	7	9	11	10	5	12	3	0
Polysorbate (Tween®) 80	10	28	6	8	10	4	5	4	1	0
Identifications: based on top scores by 3 tests by MALDI-ToF										
<i>E. cloacae</i> in 3/3 cases	36	15	2	0	0	10	4	23	12	0
<i>E. asburiae</i> in 3/3 cases	0	17	2	4	10	1	0	0	0	0
Mixed results	7	12	4	5	4	1	1	2	1	1

547 Substrates tested and metabolised by >90% of both colistin-susceptible and –resistant isolates: acetic acid, N-acetyl-D-glucosamine, N-acetyl-β-

548 D-mannosamine, adenosine, D-alanine, L-alanine, L-alanyl-glycine, L-arabinose, L-asparagine, L-aspartic acid, bromosuccinic acid, D-cellobiose,

549 citric acid, 2-deoxy adenosine, D-fructose, D-fructose-6-phosphate, fumaric acid, L-galactonic acid-γ-lactone D-galacturonic acid, D-galactose, D-

550 gluconic acid, α-D-glucose, D-glucose-1-phosphate, D-glucose-6-phosphate, D-glucuronic acid, L-glutamic acid, L-glutamine, glycerol, D,L-α-

551 glycerol phosphate, glycyl-L-glutamic acid, glycyl-L-proline, *m*-hydroxy-phenylacetic acid, *p*-hydroxy-phenylacetic acid, inosine, L-lactic acid,

552 α -D-lactose, D,L-malic acid, L-malic acid, maltose, maltotriose, D-mannitol, D-mannose, β -methyl-D-glucoside, methyl pyruvate, mucic acid, L-
553 proline, pyruvic acid, D-ribose, D-saccharic acid, L-serine, D-sorbitol, succinic acid, sucrose, thymidine, L-threonine, D-trehalose, uridine and D-
554 xylose. Compounds metabolised by <10% of both colistin-susceptible and –resistant isolates were acetoacetic acid, 2-aminoethanol, D-aspartic
555 acid, D-glucosaminic acid, glycolic acid, glyoxalic acid, α -hydroxy glutaric acid- γ -lactone, α -keto-butyric acid, D-malic acid, 1,2 propanediol,
556 propionic acid, D-psicose, m-tartaric acid, D-threonine, tricarballylic acid and tyramine.

557

558 **Table 4.** Main lipid A species present in representative isolates in relation to colistin MIC and

559 Genogroup

560

Isolate	Genogroup	Colistin MIC (mg/L)		<i>m/z</i> ion peaks observed by MALDI-TOF mass spectrometry ^b					
		Agar (BSAC)	Broth (Belfast)	1387	1797	1824	1840	2036	2063
EN104606	A	0.5	4 to 32 ^a	+	+	+	+	+	+
EN2852	A	256	512	+	+	+	+	+	+
EN104107	B	4	512	+	+	+	-	? ^c	? ^c
EN105227	B	8	>1024	+	+	+	+	+	+
EN100708	C	>32	256	+	+	+	-	-	-
EN105406	C	>32	32 to 1024 ^a	+	+	+	+	+	+
EN2692	D	16	512	+	+	+	+	+	+
NCTC/10005 ATCC13047	D	8	128	+	+	+	+	+	+
EN2720	D	4	8 to 512 ^a	+	+	+	+	+	+
EN2878	E	0.5	2	-	+	-	-	+	-
EN2889	F	0.5	2	+	+	+	+	-	+
EN104619	F	0.25	2	+	+	+	+	-	+
EN104003	F	8	4	-	-	+	-	-	-
EN115203	G	0.25	2	+	+	+	-	-	-

561

562 ^a Substantial heteroresistance / trailing end-points seen

563 ^b Proposed lipid A composition of each of the molecular ions (see fig. S1 for detailed
564 spectra):

565 1387; Tetra-acyl (3x C14:0(3-OH), 1x C14:0), 2P

566 1797; Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0), 2P

567 1824; Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 2P

568 1840; Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C14:0(3-OH), 2P

569 2036; Hepta-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0, 1x C16:0), 2P

570 2063; Hepta-acyl (4x C14:0(3-OH), 2x C14:0, 1x C16:0), 2P

571 ^c Molecular ion could not be confirmed due to background noise

572 **Figure Legends**

573 **Figure 1.** Dendrogram showing relatedness of the 95 *E. cloacae* group isolates
574 successfully sequenced together with type strains *E. cloacae*
575 NCTC10005/ATCC13047 (Genogroup D) and *E. asburiae* NCTC12123/ATCC35953
576 (Genogroup C).

577

578 Footnotes

579 A-G, Genogroups described in the text.

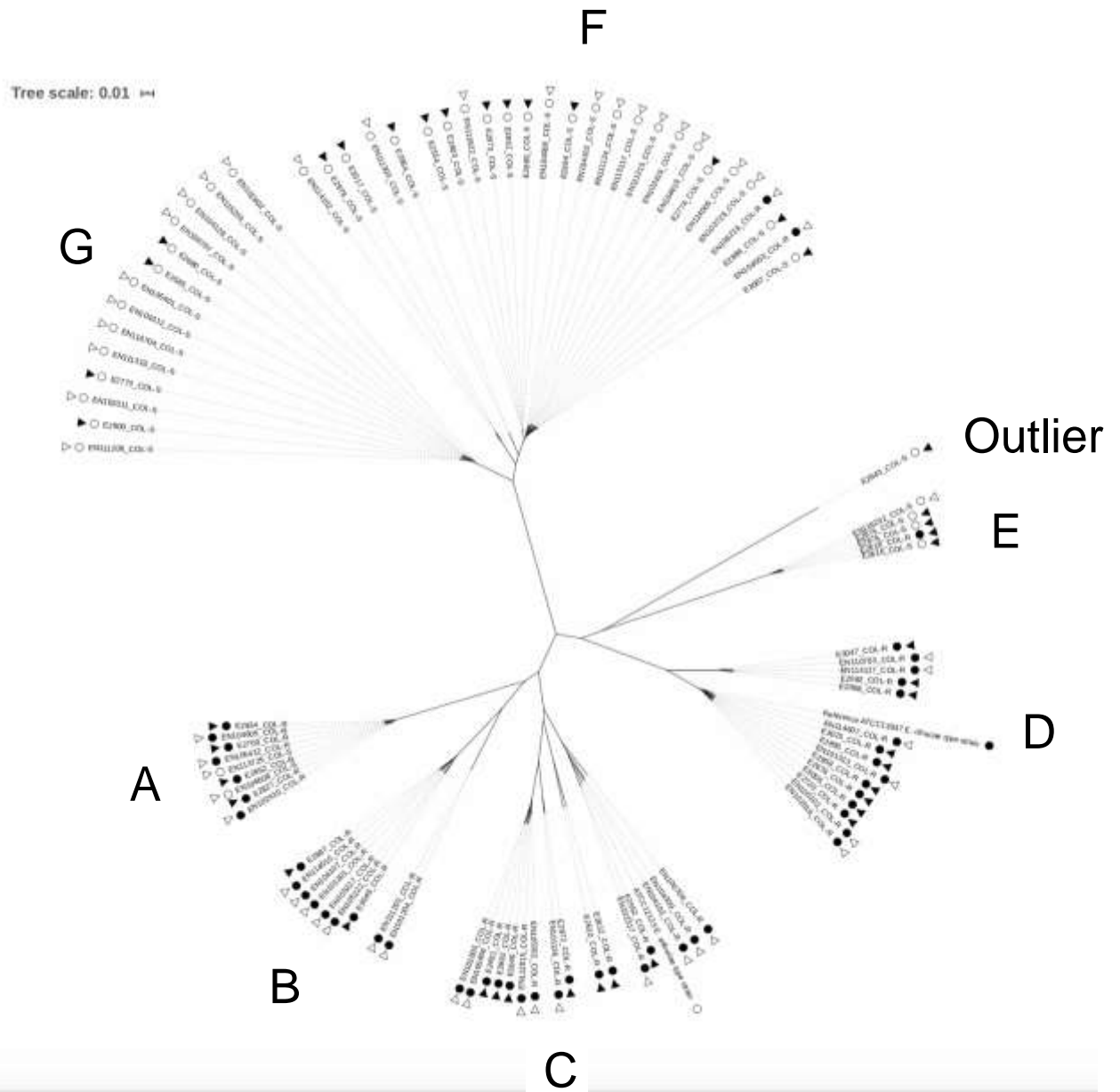
580 Open circle, colistin-susceptible isolate; solid circle, colistin-resistant isolate

581 Open triangle, respiratory isolate; solid triangle, bloodstream isolate

582

583 **Figure 2.** MALDI-TOF mass spectrometry and predicted lipid A species in the *E.*
584 *cloacae* ATCC13047 type strain. The prominent molecular ion in the spectrum (m/z
585 1824) corresponds to a di-phosphorylated hexa-acylated lipid A, which is identical to
586 that described for *Klebsiella pneumoniae*.²² Dotted squares indicate the following
587 modifications likely responsible for the observed mass shifts: I, elimination of the
588 myristoxymyristoyl group (m/z 1387) by fragmentation of m/z 1824;²³ II,
589 hydroxylation of the C'-2 myristoyl-oxo-acyl chain (m/z 1840);²² III, C'-2 lauryl-oxo-
590 acyl chain (m/z 1797); IV, palmitoylation of the C-1 acyl-oxo-acyl chain (m/z 2063);
591 V, hydroxylation of the C'-2 myristoyl-oxo-acyl chain plus palmitoylation of the C-1
592 acyl-oxo-acyl chain (m/z 2079).

593



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596

