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

4

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28 **Running Heads:** Mushtaq S *et al.* / Colistin-resistant *Enterobacter* in the UK

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33

Abstract.

Background. Polymyxins have re-entered use against problem Gram-negative bacteria. Resistance rates are uncertain, with estimates confounded by selective testing. **Methods**. The BSAC Resistance Surveillance Programme has routinely tested colistin since 2010; we reviewed data up to 2017 for relevant Enterobacterales (n=10,914). Unexpectedly frequent resistance was seen among the *Enterobacter cloacae* complex isolates (n=1749); for these, we investigated relationships to species, genome, carbon source utilisation and LPS structure. **Results**. Annual colistin resistance rates among *E. cloacae* complex isolates were 4.4% to 20%, with a rising trend among bloodstream organisms; in contrast, annual rates for *Escherichia coli, Klebsiella spp.* and *E. aerogenes* generally remained <2%. WGS split the *E. cloacae* complex isolates into 7 Genogroup clusters, designated A-G. Among isolates assigned to Genogroups A-D, 47/50 sequenced were colistin resistant, and many, from Genogroups A-C identified as *E. asburiae*. Isolates belonging to Genogroups E-G consistently identified as *E. cloacae* and were rarely (only 3/45 representatives sequenced) colistin resistant. Genogroups F and G – the predominant colistin-susceptible clusters – were metabolically distinct from other clusters, notably regarding utilisation or not of L-fucose, formic acid, D-serine, adonitol, *myo*-inositol, L-lyxose and polysorbates. LPS from resistant organisms grown without colistin pressure lacked substitutions with 4-amino-arabinose or ethanolamine but was more structurally complex, with more molecular species present. **Conclusions.** Colistin resistance is frequent in the *E. cloacae* complex and increasing among bloodstream isolates. It is associated with: (i) particular genomic and metabolic clusters, (ii) identification as *E. asburiae* and (iii) with more complex LPS architectures.

Introduction

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

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

The prevalence of colistin resistance in the UK and worldwide is uncertain and subject to detection bias because many laboratories only test the drug against bacteria resistant to standard agents. Moreover, mutational resistance is often unstable and readily lost, with the issue muddied by 'heteroresistance,'[8,9] the inadequacy of disc testing and the variability of MICS with the test method.[10] Despite these uncertainties it is clear and concerning that: (i) polymyxin-resistant mutants of carbapenemase-producing Enterobacterales can cause outbreaks[11] and (ii) that acquired *mcr* genes are widespread among *Escherichia coli* in food animals, e.g. in China.[12]

The BSAC Resistance Surveillance Programme[13] has tested colistin against Gram-negative bacteria (excepting inherently-resistant genera) since 2010/11. We 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 ≤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 Na_2HPO_4, 1.7

142 mM KH_2PO_4). The final pellets were freeze-dried overnight then lipid A was extracted

143 as previously described.[18] After de-salting, 2-μl lipid A aliquots were loaded onto the

144 polished steel target of the MS instrument, air dried and covered by 1 μl 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 $\geq$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

Discussion

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

Relating our Genogroups to the established taxonomy for *the E. cloacae* complex proved challenging. Although MALDI-ToF does not reliably identify within the *E. cloacae* complex it was striking that isolates belonging to three of the four predominantly colistin-resistant Genogroups (A, B and C but not D) were more likely to identify as *E. asburiae*, or to give a mixture of *E. asburiae* and *E. cloacae* identifications, with this likelihood rising with the colistin MIC. Conversely, isolates belonging to the predominantly susceptible Genogroups E, F and G largely identified as *E. cloacae*, as did the (mostly resistant) members of Genogroup D. The simple notion of intrinsic colistin resistance in *E. asburiae* and susceptibility in *E. cloacae* is however refuted by the literature[25] and by the type strains *E. asburiae* NCTC12123/ATCC35953 (Group C, MIC 1 mg/L) and *E. cloacae* NCTC10005/ATCC13047 (Group D, MIC 8 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

402

428   Scientific, amounting to <10% of portfolio value; **MCG, IGR, TLB, MAV, RR, MD,**

429   **CH:** nothing to declare.

430

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528 **Table 1.** Rates of resistance to colistin among Enterobacterales species collected in the BSAC Surveillance Programme
529

	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
*Enterobacter cloacae group**	**9/158**	**13/161**	**12/160**	**11/172**	**17/169**	**27/170**	**21/157**
K. aerogenes (E. aerogenes)	0/14	0/36	0/44	0/36	1/41	1/29	1/29
K. pneumoniae	2/200	4/196	1/213	1/205	**8/206**	2/186	3/164
K. oxytoca	0/56	0/55	0/53	0/55	0/57	0/58	0/57
E. coli	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
*Enterobacter cloacae group**	**21/105**	**7/93**	**10/85**	**3/68**	**9/90**	**10/90**	**8/71**
K. aerogenes (E. aerogenes)	0/34	0/42	0/40	0/22	0/23	0/35	0/32
K. pneumoniae	**4/145**	**4/166**	2/140	3/150	**4/187**	0/152	2/141
K. oxytoca	**3/71**	1/73	1/56	0/68	0/63	1/68	0/49
E. coli	**6/274**	1/239	0/250	0/255	0/244	0/230	3/277

530 Bold text indicates rates >2%
531 **Enterobacter cloacae* group comprises: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*,
532 *Enterobacter ludwigii* and *Enterobacter nimipressuralis*.
533 Until 2014/15 (respiratory) and 2015 (bacteraemia) isolates were collected from up to 40 centres across the UK and Ireland and, thereafter from
534 up to 25 centres.
535

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	*E. cloacae*	*E. asburiae*	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	5
myo-Inositol	9	42	7	9	13	12	5	4	0	1
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										
E. cloacae in 3/3 cases	36	15	2	0	0	10	4	23	12	0
E. asburiae 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

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

Genogroup

Isolate	Genogroup	Colistin MIC (mg/L) Agar (BSAC)	Broth (Belfast)	m/z ion peaks observed by MALDI-TOF mass spectrometry[b] 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	+	+	+	-	-	-

[a] Substantial heteroresistance / trailing end-points seen

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

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

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

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

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

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

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

[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

Figure 1

597 **Figure 2.**

598