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

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

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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) were colistin resistant.

50 Genogroups F and G – the predominant colistin-susceptible clusters – were
51 metabolically distinct from other clusters, notably regarding utilisation or not of L-
52 fucose, formic acid, D-serine, adonitol, *myo*-inositol, L-lyxose and polysorbates. LPS

53 from resistant organisms grown without colistin pressure lacked substitutions with 4-
54 amino-arabinose or ethanolamine but was more structurally complex, with more
55 molecular species present. **Conclusions.** Colistin resistance is frequent in *E. cloacae*

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

59 **Introduction**

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

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

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

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

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

Methods and materials

Isolate collection

Details of the BSAC Surveillance Programmes have been published previously.¹³

Briefly, these programmes collected and tested *c.* 3000 bloodstream and lower respiratory tract infection (LRTI) isolates p.a. from centres across the UK and Ireland.

The number of centres has varied from 25 to 40, with sites asked to collect fixed quotas of isolates per species group per year. There is some turnover of centres over time.

Collection for the Respiratory Programme runs from October to September, to capture winter peaks in single years; the Bacteraemia Programme runs on the calendar year.

Colistin began to be tested in October 2010 for LRTI isolates and January 2011 for bacteraemia isolates. Bacteraemia isolates were collected and tested by PHE's

Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (AMRHAI) throughout: LRTI isolates were collected and tested by Quotient

BioResearch (LGC Group, Fordam, Newmarket, UK) until the 2012/13 year and thereafter by AMRHAI.

Laboratory methods

The analysis straddles a period when species identification moved from API20E[®] strips (bioMerieux, Basingstoke, UK) to MALDI-ToF (Bruker, Bremen, Germany); this change was introduced during the 2011-12 LRTI and 2012 Bacteraemia surveillances.

In general, the direct colony method was used for MALDI-ToF, though the extraction method was employed for subsets of isolates (see Results). MALDI-ToF identification

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

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

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

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

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

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

LPS analysis

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

Statistical methods

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

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

Results

Distribution of colistin resistance in Enterobacterales

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

LPS analysis

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

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

MALDI-TOF MS analysis of the lipid A of *E. cloacae* NCTC10005/ATCC13047 (fig. 2), grown without antibiotic pressure, revealed prominent molecular ion peaks at 1387, 1797, 1840 and 2063 m/z . The 1387 m/z peak is consistent with tetra-acylated lipid A fragmentation product, while the 1824 m/z peak is consistent with hexa-myristoylated (C14) lipid A forms resembling those found in *K. pneumoniae*, *E. coli* and *Yersinia enterocolitica*.^{20,21,22} The species at 1797 m/z is consistent with a penta-myristoylated lipid A substituted with an additional C12 acyl chain (fig. 2). Modified forms of the 1824 and 1797 m/z molecular ions were also present, reflecting addition of a hydroxyl group (typically catalysed by LpxO; m/z 1840 to 1824) or a palmitate group (typically catalysed by PagP) (m/z 2063 to 1824).²⁵ These results indicate that the lipid A of strain ATCC13407 is complex and modified by 2-hydroxylation and palmitoylation. No molecular ions consistent with Ara4N or phosphoethanolamine substitutions were observed using bacteria grown in drug-free LB; an analysis of lipid A modifications under other *in vitro* growth conditions and *in vivo* will be reported elsewhere.

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

306

307 **Discussion**

308 Colistin began to be tested in the BSAC Surveillance in 2010 and we reviewed
309 subsequent resistance trends among Enterobacterales. Resistance prevalence remained
310 under 2% in almost all years for *E. coli* and *Klebsiella* (including *K. aerogenes*) but
311 was higher for bloodstream and respiratory *E. cloacae* complex isolates, with a strong
312 increasing trend for bloodstream, but not respiratory, organisms. The colistin-resistant
313 *E. cloacae* isolates were widely scattered in time and place and did not represent
314 outbreak clones, as confirmed by WGS. Rather, they largely belonged to Genogroups
315 A-D and had metabolic differences from members of Genogroups F and G, which,
316 along with Genogroup E, encompassed the great majority of colistin-susceptible *E.*
317 *cloacae*. Within Genogroups A-D, 47/49 isolates were colistin resistant compared with
318 3/45 isolates in Genogroups E-G.

319 Relating our Genogroups to the established taxonomy for *the E. cloacae*
320 complex proved challenging. Although MALDI-ToF does not reliably identify within
321 the *E. cloacae* complex it was striking that isolates belonging to three of the four
322 predominantly colistin-resistant Genogroups (A, B and C but not D) were more likely
323 to identify as *E. asburiae*, or to give a mixture of *E. asburiae* and *E. cloacae*
324 identifications, with this likelihood rising with the colistin MIC. Conversely, isolates
325 belonging to the predominantly susceptible Genogroups E, F and G largely identified
326 as *E. cloacae*, as did the (mostly resistant) members of Genogroup D. The simple notion
327 of intrinsic colistin resistance in *E. asburiae* and susceptibility in *E. cloacae* is however
328 refuted by the literature²³ and by the type strains *E. asburiae* NCTC12123 (Group C,
329 MIC 1 mg/L) and *E. cloacae* ATCC13047/NCTC10005 (Group D, MIC 8 mg/L)
330 showing the converse pattern.

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

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

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

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

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

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

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Transparency declarations:

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

530

	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

531 Bold text indicates rates >2%

532 **Enterobacter cloacae* group comprises: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*,

533 *Enterobacter ludwigii* and *Enterobacter nimipressuralis*.

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

535 up to 25 centres.

536

537 **Table 2.** Species identification within the *E. cloacae* group in relation to colistin MIC: all isolates.
538

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

539

540 ^a Omits 5 isolates for which sequencing failed

541 **Note:** From 2010 to 2012 identification was by API20E strips; subsequently identification was by MALDI-ToF using the colony method (see
542 text). Neither method gives reliable definitive identifications within the *E. cloacae* complex and ‘identifications’ were apt to vary upon repeat
543 testing; nevertheless the trend to higher proportion identified as *E. asburiae* or not identified to species level at higher colistin MIC is clear.
544

545 **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 46
<i>myo</i> -Inositol	9	42	7	9	13	12	5	4	0	1 547
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

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

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

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

551 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-

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

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

558

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

Genogroup

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	+	+	+	-	-	-

^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

Figure Legends

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

Footnotes

A-G, Genogroups described in the text.

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

Open triangle, respiratory isolate; solid triangle, bloodstream isolate

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

F

Figure S1. MALDI-TOF mass spectrometry data of lipid A profiles from selected *E. cloacae* strains.

Lipid A MALDI-TOF MS profiles of strains belonging to Genogroups A-G (See Table 4). Molecular ions m/z values are those identified for the type strain NCTC10005/ATCC13047 (see fig. 2 and Table 4). Additional m/z values are tentatively identified as follows: **1359**, Tetra-acyl (3x C14:0(3-OH), 1x C12:0), 2P; **1597**, Penta-acyl (3x C14:0(3-OH), 1x C12:0, 1x C16:0), 2P; **1745**, Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 1P; and **2036**, Hepta-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0, 1x C16:0), 2P.