

1 Dissemination of multiple carbapenem resistance genes in an in-vitro gut model simulating the
2 human colon.

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22 Running title: Carbapenem gene dissemination in gut microbiota

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31 **Abstract**

32 **Background**

33 Carbapenemase-producing Enterobacteriaceae (CPE) pose a major global health risk. Mobile genetic
34 elements account for much of the increasing CPE burden.

35 **Objective**

36 To investigate CPE colonisation and the impact of antibiotic exposure on subsequent resistance gene
37 dissemination within the gut microbiota using a model to simulate the human colon.

38 **Methods**

39 Gut models seeded with CPE-negative human faeces (screened with BioMerieux chromID® CARBA
40 SMART (Carba-Smart), Cepheid Xpert® Carba-R assay (XCR)) were inoculated with distinct
41 carbapenemase-producing *Klebsiella pneumoniae* strains (KPC, NDM) and challenged with imipenem
42 or piperacillin/tazobactam, meropenem. Resistant populations were enumerated daily on selective
43 agars (Carba-Smart); CPE genes were confirmed by PCR (XCR, Check-Direct CPE Screen for BD MAX™
44 (CDCPE)). CPE gene dissemination was tracked using PacBio® long-read sequencing.

45 **Results**

46 CPE populations increased during inoculation, plateauing at ~10x5 log₁₀cfu/mL in both models and
47 persisting throughout the experiments (>65 days), with no evidence of CPE 'washout'. Post-antibiotic
48 administration, there was evidence of interspecies plasmid transfer of blaKPC-2 (111,742bp
49 IncFII/IncR plasmid, 99% identity to pKpQIL-D2) and blaNDM-1 (~200kb IncFIB/IncFII plasmid), and
50 CPE populations rose from <0.01% to >45% of the total lactose-fermenting populations in the KPC
51 model. Isolation of a blaNDM-1 *K. pneumoniae* isolate with one chromosomal single nucleotide
52 variant versus the inoculated strain indicated clonal expansion within the model. Antibiotic
53 administration exposed a previously undetected *K. pneumoniae* encoding blaOXA-232 (KPC model).

54 **Conclusions**

55 CPE exposure can lead to colonisation, clonal expansion and resistance gene transfer within intact
56 human colonic microbiota. Furthermore, under antibiotic selective pressure, new resistant

57 populations emerge, emphasising the need for antimicrobial-exposure control.

58 **Introduction**

59 Carbapenemase producing Enterobacteriaceae (CPE) pose a major global health risk, with reports of
60 outbreaks of CPE throughout Europe¹ including the United Kingdom (UK),^{2,3} most notably in the
61 North-West of England, where a large outbreak has contributed to national spread.^{4,5} Factors driving
62 CPE acquisition and subsequent gene dissemination include increasing age,⁴ antibiotic therapy,^{4,6-8}
63 presence of intravascular access device/invasive procedure,^{6,8,9} prolonged hospital stay,^{6,7,9}
64 multiple co-morbidities⁸ and proximity to the index case.^{6,7} Such reports identify potential 'at risk'
65 individuals, but do not fully explain why some outbreaks can be contained with infection control
66 measures,¹⁰⁻¹² while others are not.^{13,14} Unlike nosocomial outbreaks of *Clostridium difficile* or
67 methicillin-resistant *Staphylococcus aureus*, CPE outbreaks often involve multiple bacterial species
68 and multiple resistance genes carried on mobile genetic elements^{12,15,16}.

69 The epidemiology of carbapenem resistance and specifically the genes encoding carbapenemases is
70 complex.^{17,18} For example, while much of the global spread of CPE is due to *K. pneumoniae*
71 harbouring KPC resistance genes,^{3,19,20} clonal populations of *K. pneumoniae* encoding VIM genes in
72 Greece,²¹ outbreaks of OXA-48 in Turkey and the UK,^{22,23} and the higher prevalence of IMP
73 resistance genes in southern Europe and Asia,²⁴ demonstrate the heterogeneous epidemiology. This
74 implies that there are factors beyond bacterial species or single exposure events that potentiate or
75 limit the spread of specific carbapenem resistance genes.

76 Susceptible bacterial populations gain resistance through mutation or via horizontal gene transfer of
77 mobile genetic elements. The human large intestine is the ideal setting for resistance gene transfer,
78 largely due to its high biomass. Here we have used an *in-vitro* human gut model, that has previously
79 successfully simulated the colon in the context of *C. difficile* infection,²⁵⁻²⁷ to assess the factors
80 favouring the dissemination of carbapenem resistance genes, and used long-read single molecule
81 real-time (SMRT) sequencing to further understand CPE resistance gene dissemination.

82 **Methods**

83 ***In-Vitro* human gut model**

84 Two triple stage human gut models were used, as previously described²⁶. Briefly, the model consists
85 of a triple stage chemostat that is seeded with faecal slurry providing the indigenous gut microbiota.
86 The model vessels are continually purged with nitrogen to maintain an anaerobic environment. The
87 pH of each vessel is controlled to replicate that found in the proximal, medial and distal sections of
88 the human colon, respectively, and the system is top fed with a complex growth media in keeping
89 with the nutritional profile of the large intestine.

90 **Faecal inoculum**

91 Faeces from healthy volunteers with no preceding antibiotic exposure (3 months) and no CPE risk
92 factors (no travel/no hospitalisation in high risk area), and which were negative on CPE screening
93 [(Biomerieux chrom ID® CARBA-SMART (Carba-Smart), Cepheid Xpert® Carba-R assay (XCR) multiplex
94 real-time PCR assay and Check-Direct CPE Screen for BD MAX™ (CD CPE) multiplex real-time PCR
95 assay], were used in this study. Faecal samples from the volunteers were pooled to produce ~50 g of
96 faeces, which was then mixed with 500 mL of pre-reduced PBS and filtered through muslin to
97 produce a smooth faecal slurry (10% w/v). Identical volunteers were used for both gut model
98 experiments.

99 **CPE strains**

100 Two distinct clinical isolates of carbapenemase producing *K. pneumoniae* were used in this study
101 both supplied by Leeds Teaching Hospitals NHS Trust, a New Delhi Metallo-β-lactamase (NDM)
102 containing strain with minimum inhibitory concentrations (MICs) of ertapenem (ERT) ≥ 32 mg/L,
103 meropenem (MER) 8 mg/L and imipenem (IMI) 1 mg/L; and a *K. pneumoniae* carbapenemase (KPC)
104 containing strain, with MICs of ERT 4 mg/L, IMI 8 mg/L, MER 4mg/L. Each gut model was inoculated
105 with a different strain that had been reconstituted on blood agar from -80°C freezer storage, and

106 sub-cultured onto CPE selective media (Carba-Smart). The presence of carbapenemase genes was
107 confirmed using XCR and CDCPE multiplex real-time PCR assays.

108 **Experimental design**

109 Two distinct gut models were used in this study to examine the behaviours of the different
110 carbapenemase gene containing *K. pneumoniae* strains (see Figure 1). Both models were primed
111 with faecal slurry and left without interventions for two weeks to allow bacterial populations to
112 stabilise.

113 The NDM gut model was challenged with increasing inocula of *K. pneumoniae* encoding *bla*_{NDM}
114 resistance genes. Inoculation commenced on day 15 of the experiment and continued for 8 days.

115 The reconstituted strain was diluted in a 10-fold series to 10⁻⁷ in peptone water. Each day, 1 mL of an
116 overnight culture (5 mL nutrient broth) of *K. pneumoniae* encoding *bla*_{NDM} was added to the model.

117 The lowest dilution (10⁻⁷) was added on day 15 and the inocula were increased daily until a neat
118 solution was added on day 22 of the experiment. Overnight cultures of the diluted *K. pneumoniae*

119 encoding *bla*_{NDM} were enumerated on MacConkey agar to ensure the inocula were as expected

120 (Supplementary material Table S1). Following the inoculation period, the model underwent a single

121 antibiotic exposure event with imipenem. The model was dosed to achieve human in vivo gut

122 intraluminal concentrations²⁸ using imipenem 11 mg/L three times daily for 5 days (day 41-45).

123 The KPC gut model was inoculated with a single inoculum of 4.9 log₁₀cfu/mL of *K. pneumoniae*

124 encoding *bla*_{KPC} genes on day 15 of the experiment. Following inoculation, the KPC model was

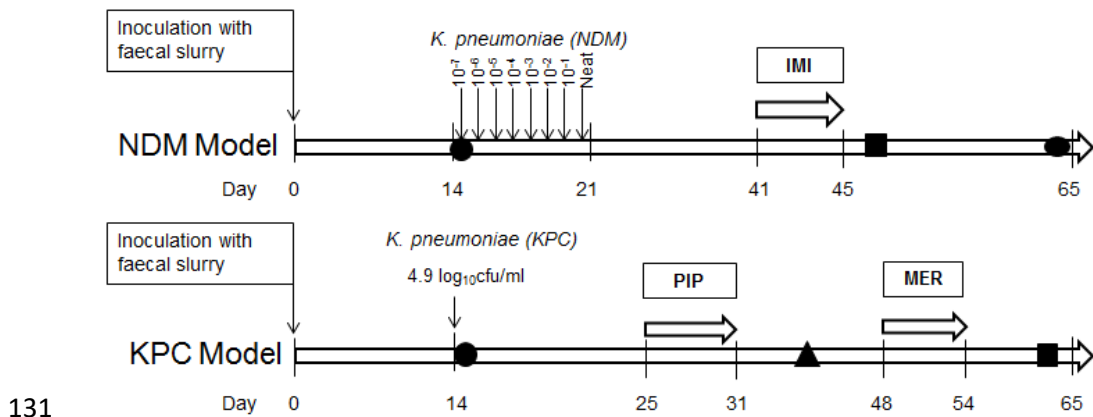
125 challenged with two separate antibiotic exposure events. Piperacillin/tazobactam (358 mg/L) was

126 instilled between days 25-31, and meropenem (11 mg/L) between days 48-54, again dosed to

127 simulate in vivo concentrations. Bacterial populations were monitored throughout (Figure 1) and

128 were sampled daily for CPE and Enterobacteriaceae populations during the inoculation and

129 antibiotic installation periods, and sporadically thereafter. The presence/absence of carbapenemase
 130 genes was monitored via the XCR assay.



131
 132 **Figure 1: Schematic of experimental design.** IMI-imipenem, PIP-piperacillin/tazobactam, MER-
 133 meropenem. Shapes represent time point at which isolates were sequenced. Circle denotes original
 134 strain inoculated into model, Square outline denotes plasmid uptake, triangle denotes strain with
 135 new resistance profile.

136 **Population monitoring**

137 Sporadic sampling of the indigenous gut populations took place before CPE inoculation. For the NDM
 138 model, twice daily sampling of CPE populations took place during the inoculation phase (day 14-21),
 139 and indigenous populations were monitored once a day. Following inoculation, both carbapenemase
 140 producing (CP) and indigenous bacterial populations were monitored daily. Both the CPE and the
 141 indigenous populations were sampled daily. Enumeration of the bacterial populations was
 142 performed by quantitative culture using Carba-Smart for CPE and MacConkey agar for indigenous
 143 populations.

144 These plates were read at 48 h using the appropriate dilution factor to allow enumeration in log₁₀
 145 cfu/mL. For CPE populations, enumeration took place 1 h post-KPC inoculation. Fifty microliters of
 146 the diluted aliquot (up to 10⁻³) was plated in triplicate on Carba-Smart and ESBL agars, incubated at
 147 37°C and read at 24 h. Again, the plates were read at a factor allowing enumeration in log₁₀ cfu/mL.
 148 The percentage of carbapenemase producing bacteria was calculated from cfu/mL on the

149 MacConkey plate (representing total lactose fermenting (TLF) population) divided by cfu/mL on the
150 Carba-Smart plate and multiplied by 100 to give a percentage of CP bacteria in the TLF bacteria.
151 For the molecular platforms (XCR, CD CPE), 50µL of neat gut model fluid was analysed the same day,
152 in accordance with the manufacturers' instructions, except that we used gut model fluid instead of a
153 rectal swab. Please see supplementary materials for further information.

154 **Selection of CPE isolates for sequencing**

155 Three isolates were chosen from each model for sequencing.

156 From the NDM model the CP *K. pneumoniae* strain inoculated into the model, designated LCC079,
157 the CP *K. pneumoniae* strain isolated at the end of the experiment, designated LCC088, and an
158 *Escherichia coli* isolate with carbapenem resistance, designated LCC081, were sequenced.

159 From the KPC model, the original CP *K. pneumoniae* strain inoculated into the model, designated
160 LCC078, an *E. coli* isolate from the end of the experiment with carbapenem resistance, designated
161 LCC096, and a *K. pneumoniae* isolate growing on the OXA side of the CARBA-SMART plate on day 40,
162 designated LCC093, were sequenced.

163 Isolates were sequenced using Illumina technology as previously described and PacBio long-read
164 sequencing at the Icahn Institute and Department of Genetics and Genomic Sciences, Mount Sinai,
165 New York.

166 **Sequencing and genetic analysis**

167 Long read SMRT sequencing and initial de novo assembly were performed as previously described²⁹
168 using the latest P6 enzyme and chemistry and a single SMRTcell on the RSII platform. Chromosomal
169 single-nucleotide variants (SNVs) were determined by mapping Illumina reads to chromosomal
170 references for *E. coli* CFT073 (AE014075.1) or *K. pneumoniae* MGH78478 (CP000647.1), as previously
171 described³⁰. To assemble the long-read sequencing data, we initially performed hybrid assembly

172 using unicycler v0.4.0³¹ in bold mode with otherwise default parameters. For the KPC and OXA-232
173 samples, this produced complete, closed assemblies (i.e. all contigs were flagged as circular). For the
174 NDM samples, the assemblies contained multiple un-circularised contigs, and visualisation of
175 mapped Illumina reads indicated likely miss-assemblies. For these samples, we therefore performed
176 a de novo long-read assembly on the PacBio subreads using HGAP3 as previously described.²⁹
177 Plasmid Inc typing was performed using the February 2018 version of the PlasmidFinder
178 Enterobacteriaceae database,³² with an identity threshold of 95% and minimum length 60%. Copy
179 number in LCC079, LCC081 and LCC088 was calculated using the unicycler assembly for LCC081,
180 which contained *bla*_{NDM-1} on the IncFIB/IncFII plasmid described below, with a single copy of the
181 repeat unit. For each sample, we mapped Illumina reads to a reference consisting of this plasmid
182 structure plus the HGAP3-assembled chromosomal contig for that sample. Mapping was performed
183 using bwa mem v0.7.12-r1039. Samtools version 1.4.1 was used to filter out supplementary
184 alignments and calculate depth of coverage. *bla*_{NDM-1} coverage relative to the plasmid backbone was
185 calculated as the median coverage across *bla*_{NDM-1} divided by the median coverage across the entire
186 plasmid sequence excluding the repeat region. Similarly, the same median coverage value for the
187 plasmid backbone was divided by the median coverage across the chromosomal contig to determine
188 plasmid coverage relative to the chromosome. Please see supplementary materials for further
189 information.

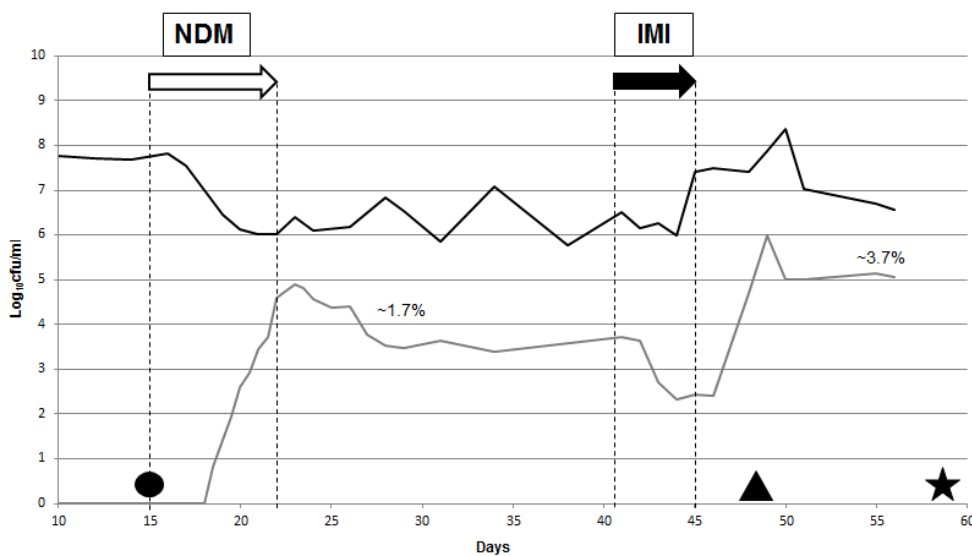
190 **Results**

191 No CPE were identified during the two-week stabilisation phase in either gut model experiment. In
192 both models, CPE populations increased after antibiotic administration, levelling at ~5 log₁₀ cfu/mL,
193 and persisted for the duration of the experiments, with no evidence of CPE washout.

194 **Plate enumeration results**

195 **NDM Model**

196 CP *K. pneumoniae* were detected after the addition of $\sim 4.9 \log_{10}$ cfu/mL (day 18); CP *K. pneumoniae*
 197 increased with increasing inocula peaking at 5-6 \log_{10} cfu/mL, before stabilising at $\sim 3.5 \log_{10}$ cfu/mL.
 198 Following antibiotic (imipenem) exposure, the CP *K. pneumoniae* population reduced, before
 199 expanding after administration stopped, finally accounting for $\sim 3.7\%$ of the TLF population. On day
 200 47 of the experiment (2 days post antibiotic exposure), we began to see the emergence of a
 201 resistant CP *E. coli* population, which appeared sporadically on the CARBA-SMART agar until the end
 202 of the experiment (Figure 1).

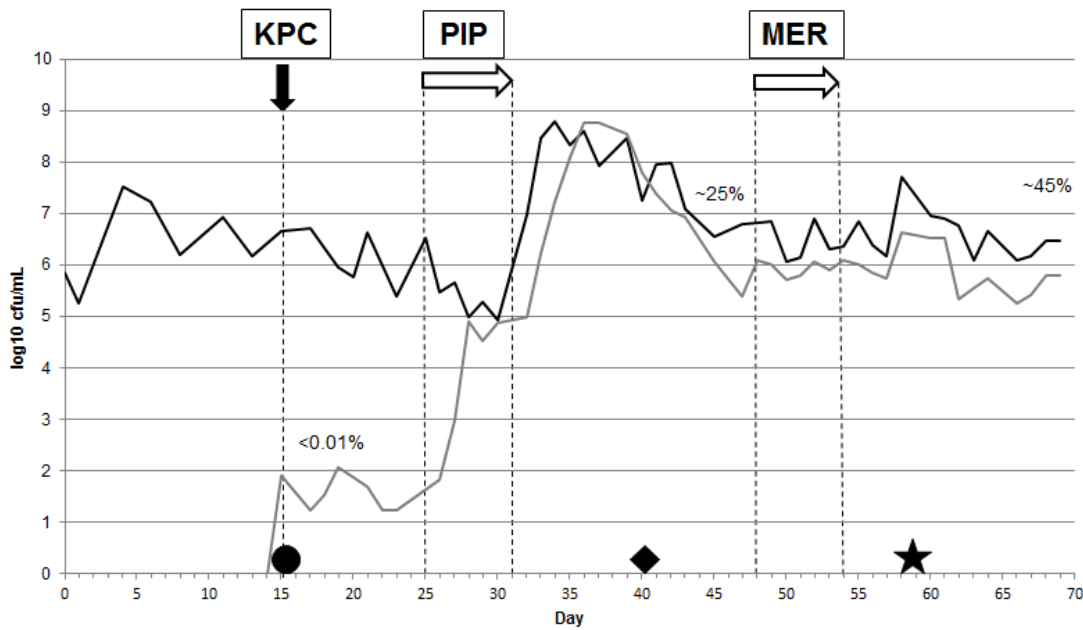


203
 204 **Figure 2: NDM model. Bacterial growth on logarithmic scale. Solid arrow-inoculation phase, open**
 205 **arrow-antibiotic instillation, NDM-Klebsiella pneumoniae with bla_{NDM} , IMI-imipenem. Black line -**
 206 **total lactose fermenting population, grey line-carbapenemase producing *K. pneumoniae*. % refers to**
 207 **resistant proportion of total Enterobacteriaceae. ● -LCC079, ▲ -LCC081, ★ -LCC088**

208 **KPC Model**

209 Post-inoculation, CP *K. pneumoniae* counts were $\sim 1-2 \log_{10}$ cfu/mL, accounting for $<0.01\%$ of the TLF
 210 population. Dosing with piperacillin/tazobactam resulted in an increase of $>8 \log_{10}$ cfu/mL in CP *K.*
 211 *pneumoniae* populations, which comprised $\sim 25\%$ of TLF population after dosing stopped. On day 35
 212 of the experiment (20 days post CP *K. pneumoniae* inoculation and 5 days post end of antibiotic
 213 administration), a CP *E. coli* emerged, peaking at $5.5 \log_{10}$ cfu/mL on day 45. Following a second
 214 antibiotic exposure with meropenem, there were further increases in carbapenemase-producing

215 populations at day 65 (end of the experiment), comprising both the CP *K. pneumoniae* and CP *E. coli*
 216 populations, which combined accounted for ~45% of the TLF population.



217

218 *Figure 3: KPC model. Bacterial growth on logarithmic scale. Solid arrow-inoculation phase, open*
 219 *arrow-antibiotic instillation KPC-Klebsiella pneumoniae with bla_{KPC}, PIP-piperacillin/tazobactam,*
 220 *MER-meropenem. Black line-total lactose fermenting population, grey line-carbapenemase*
 221 *producing K. pneumoniae. % refers to resistant proportion of total Enterobacteriaceae. ● -LCC078,*
 222 *★LCC093 - -LCC096*

223 **Long read sequencing**

224 Table one presents a summary of each bacterial isolate and its characteristics.

Model	Collection number	Day	Isolate	Subtype	Plasmid encoding carbapenemase
NDM	LCC079	15	<i>K. pneumoniae</i>	ST147	IncFIB/IncFII
	LCC088	57	<i>K. pneumoniae</i>	ST147	IncFIB/IncFII
	LCC081	48	<i>E. coli</i>	ST88	IncFIB/IncFII
KPC	LCC078	15	<i>K. pneumoniae</i>	ST661	pKpQIL-D2
	LCC096	57	<i>E. coli</i>	ST357	pKpQIL-D2
	LCC093	40	<i>K. pneumoniae</i>	ST14	ColKP3

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225 Table 1: Summary of bacterial isolates characteristics

226 **NDM Model**

227 LCC079 (*K. pneumoniae*, single-locus variant of ST147, original strain) and LCC088 (*K. pneumoniae*,
 228 same single-locus variant of ST147, isolated at the end of the experiment) differed by only one
 229 chromosomal SNV, indicating that the original inoculated strain was maintained throughout the
 230 experiment. For both these isolates, as well as LCC081 (the CP *E. coli* ST88 isolated on day 47), the
 231 *bla*_{NDM-1} gene was located on the same plasmid backbone, indicating *bla*_{NDM-1} plasmid transfer from *K.*
 232 *pneumoniae* to *E. coli* during the experiment. This plasmid represents a previously undescribed >170
 233 kb IncFIB/IncFII plasmid. Within this plasmid, the *bla*_{NDM-1} gene was located within a 6,893 bp region
 234 flanked by 1,695 bp direct repeats. There was evidence that the 6,893 bp + 1,695 bp structure was
 235 tandemly repeated: however, the exact number of repeat units in each case could not be
 236 ascertained from the long-read data, and Illumina coverage suggested variation in the number of
 237 repeat units between the three isolates (Table 2).

238 The same *bla*_{NDM-1}-containing repeat structure has previously been described in the unrelated
 239 IncHI1B/IncFIB plasmid pPMK1-NDM from a *K. pneumoniae* isolated in Nepal in 2011.³³ There was
 240 no evidence for any other shared plasmids between the *K. pneumoniae* and *E. coli* isolates.

Sample	<i>bla</i> _{NDM-1} coverage relative to plasmid backbone	Plasmid coverage relative to chromosome
LCC079	2.7	1.7
LCC081	1.6	0.9
LCC088	2.3	1.8

241 Table 2: Estimated genomic coverage of tandem repeat.

242

243

244 **KPC model**

245 LCC078 (*K. pneumoniae* ST661, original strain) and LCC096 (*E. coli* ST357, isolated on day 57) both
246 contained *bla*_{KPC-2} on an identical 111,742 bp IncFII/IncR plasmid with >99% sequence identity to
247 pKpQIL-D2⁵, indicating *bla*_{KPC} plasmid transfer from *K. pneumoniae* to *E. coli* during the experiment.
248 There were no other shared plasmids between these two isolates; LCC078 additionally contained a
249 249,604 bp IncFIB plasmid, a 4,350 bp non-typeable plasmid and a 4,000 bp Col440I plasmid, and
250 LCC096 additionally contained a 148,387 bp IncFIB/IncFIC plasmid and a 3,904 bp Col156 plasmid.
251 LCC093 (*K. pneumoniae* ST14, taken on day 40) contained *bla*_{OXA-232} on a 6,141 bp ColKP3 plasmid,
252 which was identical to a previously described *bla*_{OXA-232} plasmid from a *K. pneumoniae* ST14 isolated
253 in the US in 2013.³⁴ LCC093 additionally contained a 273,971 bp IncHI1B/IncFIB plasmid, a 211,819
254 bp IncFII/IncFIB plasmid, a 4,167 bp non-typeable plasmid and a 2,095 bp non-typeable plasmid.

255 **Discussion**

256 The *in-vitro* human gut model provides a unique insight into CPE colonisation dynamics in the colon,
257 allowing biomass enumeration of the microbiota in real time. We note that in the forerunner of our
258 gut model microbiota composition was validated against the colonic bacteria found in sudden road
259 traffic accident victims³⁵. Our model has since been used extensively to successfully predict the
260 behaviour of antibiotics in the context of *Clostridium difficile* infection, which is clearly mediated via
261 alterations to the gut microbiota.³⁶ Utilising our predictive model, we can follow changes that occur
262 in both resistant and susceptible bacterial populations and count viable bacterial populations with
263 carbapenem resistance genes. The additional long read sequencing data enabled us to track the
264 ancestral strains/plasmids as they disseminated within the simulated colon.

265 We chose to study *bla*_{KPC} and *bla*_{NDM} as these strains represent two of the 'big five' carbapenemases
266 present worldwide and are frequently encountered in clinical practice and hence freely available for
267 study. In the NDM model, increasing inocula were added over 7 days to determine the threshold at

268 which all screening tests (in triplicate) became positive. We then used our data to determine the
269 inoculum size for the KPC model, and so used a single exposure event to replicate what we believe is
270 most likely to occur in clinical practice. The selected antibiotics represent those commonly used in
271 clinical practice; the regimen of piperacillin/tazobactam followed by meropenem was utilised to
272 represent an often employed escalation of antibiotic treatment in patients who have failed to
273 respond to initial empirical (piperacillin/tazobactam) antibiotic therapy.

274 Within the NDM model, the use of increasing daily inocula demonstrated that at low levels (<4.9
275 log₁₀cfu/mL) commonly used screening methods did not detect CPE. This is a phenomenon that we
276 have observed before and is not unique to the NDM strain (unpublished data, Rooney CM, Davies K,
277 Wilcox MH, Chilton CH. HCAI research group, Leeds). For, the increasing inocula (NDM model) and
278 the single inoculum (KPC model), the resistant populations stabilised before antibiotic exposure,
279 suggesting that antibiotic induced dysbiosis not a prerequisite for CPE gut colonisation. However,
280 after antibiotic dosing, selective pressure led to a marked increase in both CPE strains. Current
281 understanding^{4, 6-8} suggests that antibiotic exposure is a risk factor for CPE colonisation, which is in
282 keeping with our findings. Nonetheless, if antibiotic selective pressure is driving already present
283 resistant populations into a detectable range, then true colonisation rates could be far higher than
284 reported.

285 Adding further weight to this argument, in the KPC model we identified on day 40 a *K. pneumoniae*
286 strain that carried *bla*_{OXA232} resistance genes, an isolate we had neither inoculated into the model nor
287 detected on screening. This isolate was only detected after antibiotic exposure and was present at
288 very low levels (sporadic colonies). Interestingly, the plasmid encoding the *bla*_{OXA232} has been
289 previously described³⁴ in a US strain carrying dual carbapenem resistance genes (NDM+ OXA),
290 although sequencing of our strain did not show both these resistance mechanisms. We hypothesize
291 that this isolate was undetectable until clonal expansion of the population occurred under antibiotic
292 selective pressure. We acknowledge that environmental contamination or presence of CPE in donor

293 samples are possible alternative explanations for our results; however, these scenarios are unlikely
294 given that no *bla*_{OXA232} resistant strains were used in the laboratory at the time of this experiment,
295 and this was not observed in the NDM model (which was primed with faeces from the same donor
296 as used in the KPC model).

297 In the NDM model, we have shown that the viable resistant population doubled after antibiotic
298 exposure; combining these results and the long read sequencing data, we have demonstrated that
299 this was due to an expanding clonal population. This provides clear evidence that the biomass of
300 resistant bacteria and the subsequent transmission risk is greatest after antibiotic selective pressure.
301 We also identified the emergence of an *E. coli* population with newly acquired carbapenem
302 resistance, similarly to the KPC model. Again, as in the KPC model, in the NDM model this population
303 shared the same plasmid as the inoculated strain, providing evidence that horizontal gene transfer
304 had taken place, promoted by antibiotic exposure, from the dominant clonal population to
305 susceptible bacterial populations, as hypothesised *in vivo*.³⁷ Factors influencing conjugation rate
306 include temperature, substrate, plasmid content, and donor and recipient strain identity.³⁸ Many of
307 these parameters are tightly controlled in our gut model that simulates the human colonic habitat.
308 Others have shown biofilm formation provides the optimal surroundings for horizontal gene transfer
309 to take place;³⁹ we intend to explore such possibilities in future CPE gut model experiments, but note
310 that biofilm does form on the inner surfaces of vessels (although this was not specifically sampled in
311 these studies).

312 The KPC model had a much higher CPE biomass than the NDM model, despite greater total inocula in
313 the NDM model. This is true for both the inoculated strain LCC078 and the newly resistant *E. coli*
314 population LCC096 when compared to LCC079 and LCC081, respectively (Figure 2-3), potentially
315 related to the different antibiotics used (piperacillin/tazobactam vs imipenem respectively) and their
316 impact on other faecal flora. We hypothesise that at a higher biomass burden within the gut we
317 would observe an increased frequency of horizontal gene transfer events. However, the exact

318 number of such events would be difficult to determine given concomitant vertical transmission. We
319 caution, nevertheless, that the CPE biomass between gut models may not be directly comparable.
320 Although the same donors have been used to increase reproducibility between models,
321 piperacillin/tazobactam dosing in the KPC model resulted in a significant increase in the CP *K.*
322 *pneumoniae* population (Figure 3), more so than imipenem exposure in the NDM model (Figure 2)
323 (reflecting the relatively low imipenem MIC of LCC079). Therefore the higher biomass of CP *K.*
324 *pneumoniae* in the KPC model is reliant on both strain characteristics and antibiotic exposure.

325 This highlights the fact that antimicrobial stewardship is crucial to containing a CPE outbreak as
326 inappropriate antibiotic prescribing may lead to large clonal expansion within the gut, leading to
327 higher biomass, frequent horizontal transfer events and subsequent spread of carbapenemase
328 producing organisms within the ward environment. Given that we were unable to detect low-level
329 CPE colonisation within the NDM gut model even when inoculating known quantities, and that
330 extended spectrum antibiotics are highly selective for carbapenemase producers, we advocate strict
331 antimicrobial stewardship policies for all patients in a ward/unit environment during a period of a
332 CPE outbreak. Slow implementation or lack of such an intervention might explain why some
333 outbreaks expand and persist, and potentially lead to endemicity in some cases.

334 **Conclusion**

335 We have used an in vitro human gut model to investigate the possible outcomes of CPE exposure
336 leading to CPE colonisation. Under antibiotic selective pressure, we have demonstrated clonal
337 expansion and, critically resistance gene transfer between species, within healthy human gut
338 microbiota. Furthermore, previously unidentified, resistant populations were found. This study
339 provides important data regarding CPE colonisation and dissemination within the gut microbiota,
340 which may have key implications for antibiotic prescribing following introduction of CPE into a
341 clinical setting and/or a CPE outbreak.

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351 **Transparency declarations**

352 MHW has received consulting fees from Abbott Laboratories, Actelion, Antabio, AiCuris, Astellas,
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362 **References**

- 363 1. Glasner C, Albiger B, Buist G et al. Carbapenemase-producing Enterobacteriaceae in Europe:
364 a survey among national experts from 39 countries, February 2013. *Euro Surveill* 2013; **18**.
- 365 2. Findlay J, Hopkins KL, Alvarez-Buylla A et al. Characterization of carbapenemase-producing
366 Enterobacteriaceae in the West Midlands region of England: 2007-14. *J Antimicrob Chemother* 2017;
367 **72**: 1054-62.
- 368 3. Woodford N, Zhang J, Warner M et al. Arrival of Klebsiella pneumoniae producing KPC
369 carbapenemase in the United Kingdom. *J Antimicrob Chemother* 2008; **62**: 1261-4.
- 370 4. Poole K, George R, Decraene V et al. Active case finding for carbapenemase-producing
371 Enterobacteriaceae in a teaching hospital: prevalence and risk factors for colonization. *J Hosp Infect*
372 2016; **94**: 125-9.
- 373 5. Doumith M, Findlay J, Hirani H et al. Major role of pKpQIL-like plasmids in the early
374 dissemination of KPC-type carbapenemases in the UK. *J Antimicrob Chemother* 2017; **72**: 2241-8.
- 375 6. Hilliquin D, Le Guern R, Thepot Seegers V et al. Risk factors for acquisition of OXA-48-
376 producing Klebsiella pneumonia among contact patients: a multicentre study. *J Hosp Infect* 2018; **98**:
377 253-9.

- 378 7. Ben-David D, Masarwa S, Navon-Venezia S et al. Carbapenem-resistant *Klebsiella*
379 pneumoniae in post-acute-care facilities in Israel. *Infect Control Hosp Epidemiol* 2011; **32**: 845-53.
- 380 8. Borer A, Saidel-Odes L, Eskira S et al. Risk factors for developing clinical infection with
381 carbapenem-resistant *Klebsiella pneumoniae* in hospital patients initially only colonized with
382 carbapenem-resistant *K pneumoniae*. *Am J Infect Control* 2012; **40**: 421-5.
- 383 9. Patel G, Huprikar S, Factor SH et al. Outcomes of carbapenem-resistant *Klebsiella*
384 pneumoniae infection and the impact of antimicrobial and adjunctive therapies. *Infect Control Hosp*
385 *Epidemiol* 2008; **29**: 1099-106.
- 386 10. Schwaber MJ, Lev B, Israeli A et al. Containment of a country-wide outbreak of carbapenem-
387 resistant *Klebsiella pneumoniae* in Israeli hospitals via a nationally implemented intervention. *Clin*
388 *Infect Dis* 2011; **52**: 848-55.
- 389 11. Lepelletier D, Berthelot P, Lucet JC et al. French recommendations for the prevention of
390 'emerging extensively drug-resistant bacteria' (eXDR) cross-transmission. *J Hosp Infect* 2015; **90**: 186-
391 95.
- 392 12. Martin J, Phan HTT, Findlay J et al. Covert dissemination of carbapenemase-producing
393 *Klebsiella pneumoniae* (KPC) in a successfully controlled outbreak: long- and short-read whole-
394 genome sequencing demonstrate multiple genetic modes of transmission. *J Antimicrob Chemother*
395 2017; **72**: 3025-34.
- 396 13. Manikal VM, Landman D, Saurina G et al. Endemic carbapenem-resistant *Acinetobacter*
397 species in Brooklyn, New York: citywide prevalence, interinstitutional spread, and relation to
398 antibiotic usage. *Clin Infect Dis* 2000; **31**: 101-6.
- 399 14. Semin-Pelletier B, Cazet L, Bourigault C et al. Challenges of controlling a large outbreak of
400 OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in a French university hospital. *J Hosp*
401 *Infect* 2015; **89**: 248-53.
- 402 15. Cerqueira GC, Earl AM, Ernst CM et al. Multi-institute analysis of carbapenem resistance
403 reveals remarkable diversity, unexplained mechanisms, and limited clonal outbreaks. *Proc Natl Acad*
404 *Sci USA* 2017; **114**: 1135-40.
- 405 16. Sheppard AE, Stoesser N, Wilson DJ et al. Nested Russian Doll-Like Genetic Mobility Drives
406 Rapid Dissemination of the Carbapenem Resistance Gene blaKPC. *Antimicrob Agents Chemother*
407 2016; **60**: 3767-78.
- 408 17. van Duin D, Doi Y. The global epidemiology of carbapenemase-producing Enterobacteriaceae.
409 *Virulence* 2017; **8**: 460-9.
- 410 18. Codjoe FS, Donkor ES. Carbapenem Resistance: A Review. *Medical Sciences* 2018; **6**: 1.
- 411 19. Yigit H, Queenan AM, Anderson GJ et al. Novel carbapenem-hydrolyzing beta-lactamase,
412 KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*
413 2001; **45**: 1151-61.
- 414 20. Kitchel B, Sundin DR, Patel JB. Regional dissemination of KPC-producing *Klebsiella*
415 pneumoniae. *Antimicrob Agents Chemother* 2009; **53**: 4511-3.
- 416 21. Ikonomidis A, Tokatlidou D, Kristo I et al. Outbreaks in distinct regions due to a single
417 *Klebsiella pneumoniae* clone carrying a bla VIM-1 metallo- β -lactamase gene. *J Clin Microbiol*
418 2005; **43**: 5344-7.
- 419 22. Carrer A, Poirel L, Eraksoy H et al. Spread of OXA-48-positive carbapenem-resistant *Klebsiella*
420 pneumoniae isolates in Istanbul, Turkey. *Antimicrob Agents Chemother* 2008; **52**: 2950-4.
- 421 23. Thomas CP, Moore LS, Elamin N et al. Early (2008-2010) hospital outbreak of *Klebsiella*
422 pneumoniae producing OXA-48 carbapenemase in the UK. *Int J Antimicrob Agents* 2013; **42**: 531-6.
- 423 24. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing
424 Enterobacteriaceae. *Emerg Infect Dis* 2011; **17**: 1791-8.
- 425 25. Best EL, Freeman J, Wilcox MH. Models for the study of *Clostridium difficile* infection. *Gut*
426 *Microbes* 2012; **3**: 145-67.

- 427 26. Freeman J, O'Neill FJ, Wilcox MH. Effects of cefotaxime and desacetylcefotaxime upon
428 Clostridium difficile proliferation and toxin production in a triple-stage chemostat model of the
429 human gut. *J Antimicrob Chemother* 2003; **52**: 96-102.
- 430 27. Chilton CH, Crowther GS, Baines SD et al. In vitro activity of cadazolid against clinically
431 relevant Clostridium difficile isolates and in an in vitro gut model of C. difficile infection. *J Antimicrob*
432 *Chemother* 2014; **69**: 697-705.
- 433 28. Mayer M, Tophof C, Opferkuch W. Bile levels of imipenem in patients with t-drain following
434 the administration of Imipenem/Cilastatin. *Infection* 1988; **16**: 225-8.
- 435 29. Mathers AJ, Stoesser N, Sheppard AE et al. Klebsiella pneumoniae carbapenemase (KPC)-
436 producing K. pneumoniae at a single institution: insights into endemicity from whole-genome
437 sequencing. *Antimicrob Agents Chemother* 2015; **59**: 1656-63.
- 438 30. Stoesser N, Sheppard AE, Peirano G et al. Genomic epidemiology of global Klebsiella
439 pneumoniae carbapenemase (KPC)-producing Escherichia coli. *Sci Rep* 2017; **7**: 5917.
- 440 31. Wick RR, Judd LM, Gorrie CL et al. Unicycler: Resolving bacterial genome assemblies from
441 short and long sequencing reads. *PLoS Comput Biol* 2017; **13**: e1005595.
- 442 32. Carattoli A, Zankari E, Garcia-Fernandez A et al. In silico detection and typing of plasmids
443 using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 2014;
444 **58**: 3895-903.
- 445 33. Stoesser N, Giess A, Batty EM et al. Genome sequencing of an extended series of NDM-
446 producing Klebsiella pneumoniae isolates from neonatal infections in a Nepali hospital characterizes
447 the extent of community- versus hospital-associated transmission in an endemic setting. *Antimicrob*
448 *Agents Chemother* 2014; **58**: 7347-57.
- 449 34. Doi Y, Hazen TH, Boitano M et al. Whole-genome assembly of Klebsiella pneumoniae
450 coproducing NDM-1 and OXA-232 carbapenemases using single-molecule, real-time sequencing.
451 *Antimicrob Agents Chemother* 2014; **58**: 5947-53.
- 452 35. Macfarlane GT, Macfarlane S, Gibson GR. Validation of a Three-Stage Compound Continuous
453 Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of
454 Bacteria in the Human Colon. *Microb Ecol* 1998; **35**: 180-7.
- 455 36. Baines SD, Freeman J, Wilcox MH. Effects of piperacillin/tazobactam on Clostridium difficile
456 growth and toxin production in a human gut model. *J Antimicrob Chemother* 2005; **55**: 974-82.
- 457 37. Borgia S, Lastovetska O, Richardson D et al. Outbreak of carbapenem-resistant
458 enterobacteriaceae containing blaNDM-1, Ontario, Canada. *Clin Infect Dis* 2012; **55**: e109-17.
- 459 38. Hardiman CA, Weingarten RA, Conlan S et al. Horizontal Transfer of Carbapenemase-
460 Encoding Plasmids and Comparison with Hospital Epidemiology Data. *Antimicrob Agents Chemother*
461 2016; **60**: 4910-9.
- 462 39. Madsen JS, Burmolle M, Hansen LH et al. The interconnection between biofilm formation
463 and horizontal gene transfer. *FEMS Immunol Med Microbiol* 2012; **65**: 183-95.

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