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

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Running title: Carbapenem gene dissemination in gut microbiota

Abstract

Background

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

Objective

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

Methods

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

Results

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

Conclusions

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

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

Introduction

Carbapenemase producing Enterobacteriaceae (CPE) pose a major global health risk, with reports of outbreaks of CPE throughout Europe[1] including the United Kingdom (UK),[2, 3] most notably in the North-West of England, where a large outbreak has contributed to national spread.[4, 5] Factors driving CPE acquisition and subsequent gene dissemination include increasing age,[4] antibiotic therapy,[4, 6-8] presence of intravascular access device/invasive procedure, [6, 8, 9] prolonged hospital stay,[6, 7, 9] multiple co-morbidities[8] and proximity to the index case.[6, 7] Such reports identify potential 'at risk' individuals, but do not fully explain why some outbreaks can be contained with infection control measures,[10-12] while others are not.[13, 14] Unlike nosocomial outbreaks of *Clostridium difficile* or methicillin-resistant *Staphylococcus aureus*, CPE outbreaks often involve multiple bacterial species and multiple resistance genes carried on mobile genetic elements[12, 15, 16].

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

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

Methods

In-Vitro human gut model

Two triple stage human gut models were used, as previously described[26]. Briefly, the model consists of a triple stage chemostat that is seeded with faecal slurry providing the indigenous gut microbiota. The model vessels are continually purged with nitrogen to maintain an anaerobic environment. The pH of each vessel is controlled to replicate that found in the proximal, medial and distal sections of the human colon, respectively, and the system is top fed with a complex growth media in keeping with the nutritional profile of the large intestine.

Faecal inoculum

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

CPE strains

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

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

Experimental design

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

The NDM gut model was challenged with increasing inocula of *K. pneumoniae* encoding bla_{NDM} resistance genes. Inoculation commenced on day 15 of the experiment and continued for 8 days. The reconstituted strain was diluted in a 10-fold series to 10^{-7} in peptone water. Each day, 1 mL of an overnight culture (5 mL nutrient broth) of *K. pneumoniae* encoding bla_{NDM} was added to the model. The lowest dilution (10^{-7}) was added on day 15 and the inocula were increased daily until a neat solution was added on day 22 of the experiment. Overnight cultures of the diluted *K. pneumoniae* encoding bla_{NDM} were enumerated on MacConkey agar to ensure the inocula were as expected (Supplementary material Table S1). Following the inoculation period, the model underwent a single antibiotic exposure event with imipenem. The model was dosed to achieve human in vivo gut intraluminal concentrations[28] using imipenem 11 mg/L three times daily for 5 days (day 41-45).

The KPC gut model was inoculated with a single inoculum of 4.9 $\log_{10}$cfu/mL of *K. pneumoniae* encoding bla_{KPC} genes on day 15 of the experiment. Following inoculation, the KPC model was challenged with two separate antibiotic exposure events. Piperacillin/tazobactam (358 mg/L) was instilled between days 25-31, and meropenem (11 mg/L) between days 48-54, again dosed to simulate in vivo concentrations. Bacterial populations were monitored throughout (Figure 1) and were sampled daily for CPE and Enterobacteriaceae populations during the inoculation and

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

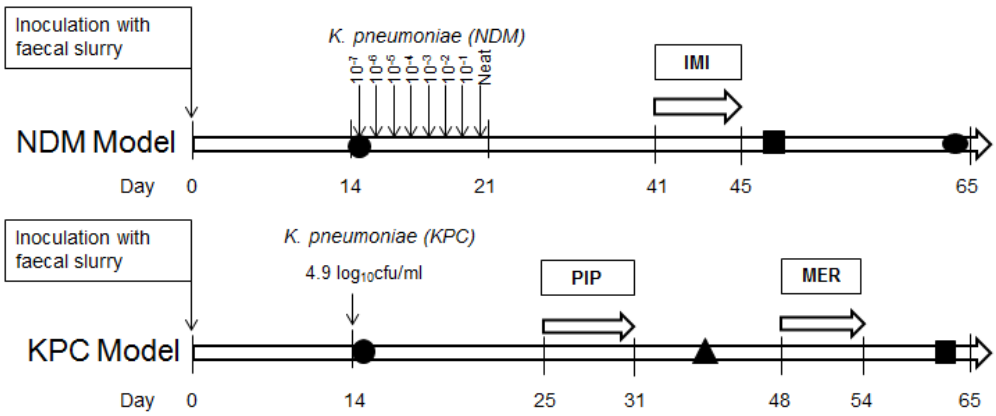

Figure 1: Schematic of experimental design. IMI-imipenem, PIP-piperacillin/tazobactam, MER-meropenem. Shapes represent time point at which isolates were sequenced. Circle denotes original strain inoculated into model, Square outline denotes plasmid uptake, triangle denotes strain with new resistance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

Population monitoring

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

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

MacConkey plate (representing total lactose fermenting (TLF) population) divided by cfu/mL on the Carba-Smart plate and multiplied by 100 to give a percentage of CP bacteria in the TLF bacteria.

For the molecular platforms (XCR, CD CPE), 50µL of neat gut model fluid was analysed the same day, in accordance with the manufacturers' instructions, except that we used gut model fluid instead of a rectal swab. Please see supplementary materials for further information.

Selection of CPE isolates for sequencing

Three isolates were chosen from each model for sequencing.

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

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

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

Sequencing and genetic analysis

Long read SMRT sequencing and initial de novo assembly were performed as previously described[29] using the latest P6 enzyme and chemistry and a single SMRTcell on the RSII platform. Chromosomal single-nucleotide variants (SNVs) were determined by mapping Illumina reads to chromosomal references for *E. coli* CFT073 (AE014075.1) or *K. pneumoniae* MGH78478 (CP000647.1), as previously described[30].To assemble the long-read sequencing data, we initially performed hybrid assembly

using unicycler v0.4.0[31] in bold mode with otherwise default parameters. For the KPC and OXA-232 samples, this produced complete, closed assemblies (i.e. all contigs were flagged as circular). For the NDM samples, the assemblies contained multiple un-circularised contigs, and visualisation of mapped Illumina reads indicated likely miss-assemblies. For these samples, we therefore performed a de novo long-read assembly on the PacBio subreads using HGAP3 as previously described.[29] Plasmid Inc typing was performed using the February 2018 version of the PlasmidFinder Enterobacteriaceae database, [32] with an identity threshold of 95% and minimum length 60%. Copy number in LCC079, LCC081 and LCC088 was calculated using the unicycler assembly for LCC081, which contained *bla*$_{NDM-1}$ on the IncFIB/IncFII plasmid described below, with a single copy of the repeat unit. For each sample, we mapped Illumina reads to a reference consisting of this plasmid structure plus the HGAP3-assembled chromosomal contig for that sample. Mapping was performed using bwa mem v0.7.12-r1039. Samtools version 1.4.1 was used to filter out supplementary alignments and calculate depth of coverage. *bla*$_{NDM-1}$ coverage relative to the plasmid backbone was calculated as the median coverage across *bla*$_{NDM-1}$ divided by the median coverage across the entire plasmid sequence excluding the repeat region. Similarly, the same median coverage value for the plasmid backbone was divided by the median coverage across the chromosomal contig to determine plasmid coverage relative to the chromosome. Please see supplementary materials for further information.

Results

No CPE were identified during the two-week stabilisation phase in either gut model experiment. In both models, CPE populations increased after antibiotic administration, levelling at ~5 $\log_{10}$ cfu/mL, and persisted for the duration of the experiments, with no evidence of CPE washout.

Plate enumeration results

NDM Model

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

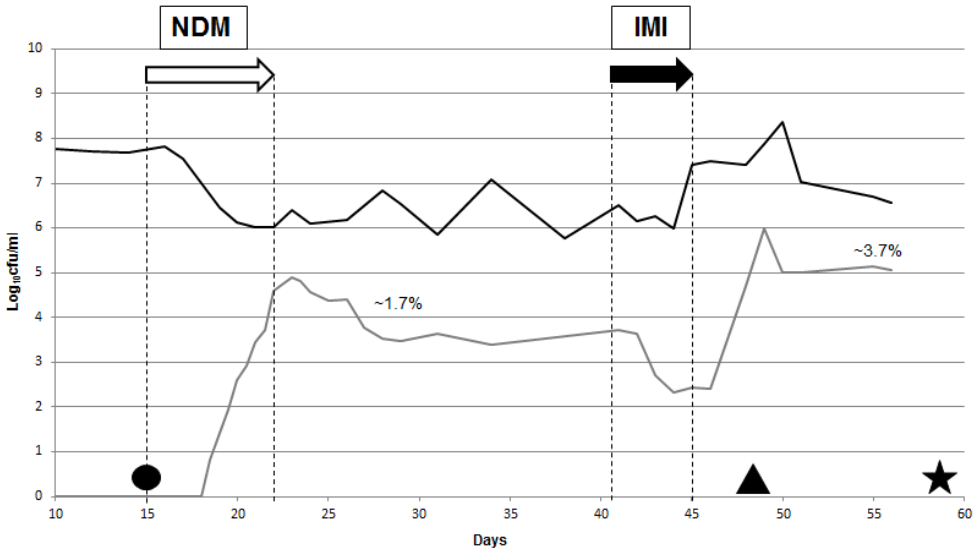

Figure 2: NDM model. Bacterial growth on logarithmic scale. Solid arrow-inoculation phase, open arrow-antibiotic instillation, NDM-Klebsiella pneumoniae with bla_{NDM}, IMI-imipenem. Black line - total lactose fermenting population, grey line-carbapenemase producing K. pneumoniae. % refers to resistant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

KPC Model

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

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

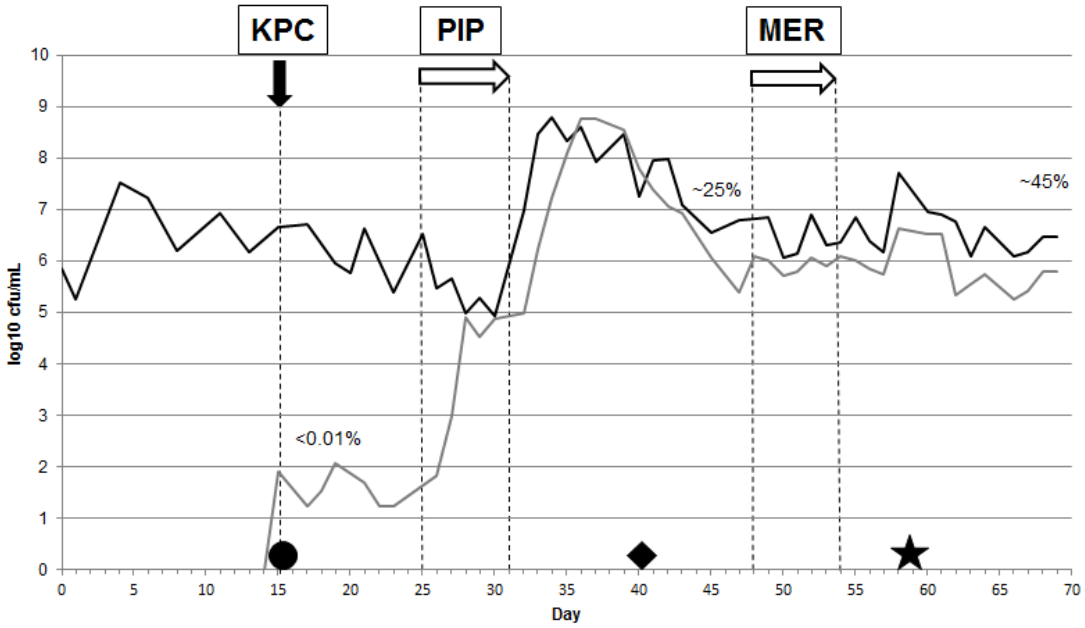

Figure 3: KPC model. Bacterial growth on logarithmic scale. Solid arrow-inoculation phase, open arrow-antibiotic instillation KPC-Klebsiella pneumoniae with bla$_{KPC}$, PIP-piperacillin/tazobactam, MER-meropenem. Black line-total lactose fermenting population, grey line-carbapenemase producing K. pneumoniae.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

Long read sequencing

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

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

Table 1: Summary of bacterial isolates characteristics

NDM Model

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

The same bla_{NDM-1}-containing repeat structure has previously been described in the unrelated IncHI1B/IncFIB plasmid pPMK1-NDM from a *K. pneumoniae* isolated in Nepal in 2011. [33] There was no evidence for any other shared plasmids between the *K. pneumoniae* and *E. coli* isolates.

Sample	**bla_{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

Table 2: Estimated genomic coverage of tandem repeat.

KPC model

LCC078 (*K. pneumoniae* ST661, original strain) and LCC096 (*E. coli* ST357, isolated on day 57) both contained bla_{KPC-2} on an identical 111,742 bp IncFII/IncR plasmid with >99% sequence identity to pKpQIL-D2 [5], indicating bla_{KPC} plasmid transfer from *K. pneumoniae* to *E. coli* during the experiment. There were no other shared plasmids between these two isolates; LCC078 additionally contained a 249,604 bp IncFIB plasmid, a 4,350 bp non-typeable plasmid and a 4,000 bp Col440I plasmid, and LCC096 additionally contained a 148,387 bp IncFIB/IncFIC plasmid and a 3,904 bp Col156 plasmid.

LCC093 (*K. pneumoniae* ST14, taken on day 40) contained $bla_{OXA-232}$ on a 6,141 bp ColKP3 plasmid, which was identical to a previously described $bla_{OXA-232}$ plasmid from a *K. pneumoniae* ST14 isolated in the US in 2013. [34] LCC093 additionally contained a 273,971 bp IncHI1B/IncFIB plasmid, a 211,819 bp IncFII/IncFIB plasmid, a 4,167 bp non-typeable plasmid and a 2,095 bp non-typeable plasmid.

Discussion

The *in-vitro* human gut model provides a unique insight into CPE colonisation dynamics in the colon, allowing biomass enumeration of the microbiota in real time. We note that in the forerunner of our gut model microbiota composition was validated against the colonic bacteria found in sudden road traffic accident victims[35]. Our model has since been used extensively to successfully predict the behaviour of antibiotics in the context of *Clostridium difficile* infection, which is clearly mediated via alterations to the gut microbiota.[36] Utilising our predictive model, we can follow changes that occur in both resistant and susceptible bacterial populations and count viable bacterial populations with carbapenem resistances genes. The additional long read sequencing data enabled us to track the ancestral strains/plasmids as they disseminated within the simulated colon.

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

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

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

Adding further weight to this argument, in the KPC model we identified on day 40 a *K. pneumoniae* strain that carried bla_{OXA232} resistance genes, an isolate we had neither inoculated into the model nor detected on screening. This isolate was only detected after antibiotic exposure and was present at very low levels (sporadic colonies). Interestingly, the plasmid encoding the bla_{OXA232} has been previously described[34] in a US strain carrying dual carbapenem resistance genes (NDM+ OXA), although sequencing of our strain did not show both these resistance mechanisms. We hypothesize that this isolate was undetectable until clonal expansion of the population occurred under antibiotic selective pressure. We acknowledge that environmental contamination or presence of CPE in donor

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

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

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

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

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

Conclusion

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

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Transparency declarations

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