The scope of antimicrobial resistance in Residential Aged Care Facilities determined through analysis of *Escherichia coli* and the total wastewater resistome

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ABSTRACT

High and often inappropriate antibiotic use has been documented for residential aged care facilities (RACFs). As a result, RACFs represent selective environments favoring the development of antimicrobial resistance (AMR). However, surveillance of the prevalence and scope of AMR in RACFs is limited. Here, wastewater-based epidemiology encompassing culture-based methods in combination with whole genome sequencing and metagenomics allowed for the in-depth analysis of the prevalence of antimicrobial-resistant *Escherichia coli*

in two RACFs and one Retirement facility. Wastewater was collected at five different time points over 18 months. From these, *E. coli* were isolated and assessed for phenotypic and genotypic resistance. The antimicrobial resistome of each wastewater sample was also determined. A comparison of facilities revealed a higher prevalence of AMR and multidrug-resistant *E. coli* observed in one RACF. The international high-risk *E. coli* clone, ST131, carrying CTX-M-like extended-spectrum beta-lactamases was exclusively isolated from this facility. A high proportion of these isolates were classified as belonging to phylogroups B2 and D, associated with virulent extra-intestinal *E. coli* infections. The resistome of this facility also revealed a higher prevalence of mobile resistance genes such as *sul1* and *sul2*, conferring sulfamethoxazole resistance, the plasmid-mediated quinolone-resistance (PMRQ) gene *qnrS*, and *qacEdelta1*, that confers biocide resistance. High fluoroquinolone resistance rates were observed in all three sample sites despite measures in place limiting the use of this class of antibiotics. The findings of this study illustrate that RACFs are highly selective environments that require measures to limit AMR development, potentially through antimicrobial stewardship.

IMPORTANCE

Antimicrobial resistance (AMR) is a global threat that imposes a heavy burden on our health and economy. Residential aged care facilities (RACFs), where frequent inappropriate antibiotic use creates a selective environment that promotes the development of bacterial resistance significantly contribute to this problem. We used wastewater-based epidemiology to provide a holistic whole-facility assessment and comparison of antimicrobial resistance in two RACFs and a retirement village. Resistant *E. coli*, a common and oftentimes problematic pathogen within RACFs was isolated from the wastewater and the phenotypic and genotypic

AMR was determined for all isolates. We observed a high prevalence of an international high-risk clone, carrying an extended-spectrum beta-lactamase in one facility. Analysis of the entire resistome also revealed a greater number of mobile resistance genes in this facility. Finally, both facilities displayed high fluoroquinolone resistance rates – a worrying trend seen globally despite measures in place aimed at limiting their use.

KEYWORDS Antimicrobial resistance, wastewater-based epidemiology, *Escherichia coli*, multidrug resistance, metagenomics

INTRODUCTION

Antimicrobial resistance (AMR) is an urgent and global threat [1], with recent reports estimating that approximately 4.95 million AMR associated deaths occurred in 2019 [2]. Continual AMR development is also speculated to add to the global economic burden, as projections indicate that 300 million people will lose their lives to AMR over the next 35 years, resulting with a loss of 60 to 100 trillion USD worth of economic output [1]. In addition to increased mortality rates, AMR can lead to increased duration of illness particularly for those who are immunocompromised [3, 4]. In the elderly population, where individuals are at an increased risk of incidence and severity of bacterial infections, AMR bacteria contribute significantly to the burden of infection, with infectious diseases accounting for one third of all deaths in those aged 65 years and over [5-7].

A significant contributor to the development and spread of AMR is the overuse and misuse of antibiotics, which promotes the selection of AMR bacteria [8-11]. Residential aged care facilities (RACFs) exemplify the overuse and frequent inappropriate use of antibiotics [12-14]. This coupled with the vulnerability of the residents resulting from comorbidities, a

compromised immune system, close living proximities, hospital visits and frequent healthcare worker contact, facilitate the spread of resistant bacteria and promote a high infection burden among the residents [15-17]. As such, RACFs and residents themselves have been identified as important reservoirs for the development of emerging AMR and multidrug resistant (MDR) bacteria [18].

Surveillance has often been limited to healthcare setting such as hospitals, where pathogens isolated from clinical infections are reported [19, 20]. Surveillance studies of these in wastewater from RACFs are scant [21]. Studies assessing AMR in RACFs are resident focussed and, although vital, are fraught with limitations regarding the number of residents included in each study, and as such, do not always represent the facility as a whole. Nonetheless, such studies have reported a high prevalence of MDR extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* [22, 23]. A point prevalence survey of three Melbourne based RACFs revealed that 27 % of residents were colonised with ESBL-producing *E. coli*, which were clonal in nature [22]. An increasing prevalence of ESBL-producing *E. coli* has also been observed in aged care facilities in Germany [24, 25], Japan [26] and the Netherlands [27]. ESBL-producing ST131 *E. coli* were also observed in aged care facilities in Germany [24] and Portugal [28]. This clonal type, also referred to as a worldwide pandemic clone [29], has spread rapidly throughout different parts of the world since its discovery in 2008 [30]. In addition to ESBL expressing ST131, a UK study has also found fluoroquinolone-resistant but ESBL lacking ST131 strains carrying the plasmid-mediated *aac(6')-Ib-cr* gene, which reduced susceptibility to ciprofloxacin [31], suggesting a split and continual emergence of ST131. In Australia, rates of *E. coli* found to be resistant to beta-lactams such as ceftriaxone and fluoroquinolones such as ciprofloxacin continue to increase, despite restrictions being in place to limit access to these agents [32]. The

prevalence of these underscores the importance of surveillance studies in RACF, with the use of wastewater-based surveillance offering an insight into the facility as whole.

Therefore, the aim of this study was to utilise wastewater-based epidemiology, consisting of microbial culturing, whole genome sequencing and metagenomics to investigate AMR within two Adelaide RACFs. The use of wastewater in this thorough investigation has circumvented one of the most common limitations in studies of AMR in RACFs - low recruitment numbers of participants - and allowed for a holistic approach to the assessment of AMR in entire RACFs. This study demonstrates the importance of surveillance studies focussing on AMR within RACFs. Housing a vulnerable older population, the development of bacterial resistance within these facilities contributes to a growing burden of infection for which treatment may become more limited. As an ageing population, with 1 in 6 estimated to be aged over 60 years old by 2030 [33], the development of AMR within RACFs, as such, is area of great concern. This study exemplifies a novel approach to the assessment of AMR within RACFs, as the use of wastewater, microbial culturing, whole genome sequencing and metagenomics to assess the development of microbial resistance has not previously been undertaken in this setting. The inclusion of one facility which has implemented an antimicrobial stewardship (AMS) program and another which has not, also allowed for an assessment of the effect that an AMS program may have on the prevalence of AMR bacteria within a facility.

MATERIALS AND METHODS

Sampling

Wastewater was sampled from two RACFs (Facility 1 with 170 beds and Facility 2 with 70 beds) and one Retirement village (38 apartments with residents of similar age to those from

the RACFs) in Adelaide. All sites are managed by the same service provider and are located within a 20 km radius of each other. Of the two RACFs Facility 2 has had an AMS program (Supplemental 1) implemented for 3 years prior to this study, whilst Facility 1 does not have AMS program implemented. As both aged care facilities are managed by the same care provider, they are comparable in the amenities and care provided. Both facilities have long-term residents in addition to respite care.

Wastewater samples were collected at approximately three-month intervals at five different time points from October 2019 to February 2021. Grab samples of approximately 200 ml were collected every hour over a 10-hour period, with collection starting at 7 am to capture the morning routine. These were collected from an access point which captured all of wastewater from the RACF, before it flowed into the main sewage system. Samples were then stored on ice and pooled for analysis. Sampled wastewater was transported to the laboratory on ice, stored at 4 °C and analyzed on the day of collection.

Isolation of *Escherichia coli*

AMR *E. coli* isolates were screened on selective and differential plates. For isolation of *E. coli* from wastewater, 100 µl wastewater (neat and 1:10 dilution) was plated onto selective and differential plates. Presumptive identification of *E. coli* was determined using two sets of Brilliance™ *E. coli*/coliform (CM0956, Oxoid, Australia) plates supplemented with either 1 mg/L ceftazidime or 0.5 mg/L of ciprofloxacin and Tergitol 7 (CM0793, Oxoid) plates supplemented with 0.5 mg/L ciprofloxacin. Cultures were incubated at 37 °C for 18 h. Following colony purification, identification was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH, Bremen, Germany). Confirmed *E. coli* isolates were stored at -80 °C in Tryptone Soya broth (CM0129, Oxoid) supplemented with 20 % (v/v) glycerol.

Antimicrobial susceptibility testing

Antibiotics assessed in this study included cefepime, ceftazidime, piperacillin-tazobactam, gentamicin, meropenem, trimethoprim-sulfamethoxazole, colistin (ChemSupply, Australia) and ciprofloxacin (Sigma-Aldrich, Australia). The Minimum Inhibitory Concentration (MIC) of each antibiotic assessed in this study was determined using the broth dilution method as described by the ISO standard [34-36]. Briefly, a 96 well microtiter plate was prepared with twofold serial dilutions of the antimicrobial agent. Cultures at an OD_{600} of 0.0025 nm were used as an inoculum using Mueller Hinton broth as a growth medium. *E. coli* ATCC 25922 was used as a quality control strain. The 96 well microtiter plates were incubated at 37 °C with 120 rpm shaking for 16 h. Resistance/susceptibility was determined by measuring bacterial growth by optical density at 600 nm using a Thermofisher Multiskan FC photometer (Thermofisher, Australia) using *E. coli* ATCC25922 for quality control. Epidemiological cutoff values, as reported by the European Committee on Antimicrobial Susceptibility

Testing (https://mic.eucast.org/search/) were used to distinguish wild type from non-wild type isolates. In this manuscript, the terms "susceptible" and "resistant" are used to refer to "wild type" and "non-wild type". Moreover, resistance to at least one antimicrobial agent in ≥ 3 antimicrobial categories were considered as MDR [37]. To ensure assessment was not carried out on duplicate *E. coli* isolates, the resistance profile of all *E. coli* was evaluated. Isolates observed to have the same resistance profile and that were isolated from the same site at the same time-point were subsequently eliminated from further assessment.

DNA extraction

Genomic extraction for whole genome sequencing

Following colony purification, a single colony was incubated in LB broth and grown at 37 ºC overnight. Bacterial cells were obtained by centrifugation (5000 x g, 5 min) of 5 ml of culture broth. Genomic DNA was extracted from the pellet using the MN NucleoSpin®Microbial DNA kit (Machery-Nagel GmbH and Co.KG, Duren, Germany), following manufacturer's instructions. DNA quality and quantity were assessed using a Cytation5 imaging reader (BioTek instruments, Winoosi Vermont, USA). Extracted genomic DNA was stored at -20 °C [35, 38].

Genomic extraction for shotgun metagenomic sequencing

Wastewater samples (100 ml) were initially filtered through an 8 µm cellulose nitrate filter to remove debris and then through a 0.2 µm pore size cellulose nitrate filter (both from Sartorius, Goettingen, Germany). Following filtration, genomic DNA was extracted from the membranes using the DNeasy® PowerWater kit (Qiagen, Hilden, Germany), following manufacturer's instructions. DNA extracts were stored at -20 °C prior to being analyzed by metagenomic sequencing. DNA quantity and quality were assessed spectrophotometrically

using the Cytation5 imaging reader (BioTek instruments, Winoosi Vermont, USA) and the Take3 MicroVolume plate.

Whole genome sequencing and bioinformatic analysis

Whole genome sequencing (WGS) was performed at SA Pathology (Adelaide, Australia) using the Illumia NextSeq platform. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit (Illumina Inc., USA) as per manufacturer's instructions. WGS was performed on the Illumina NextSeq 550 platform with the NextSq 500/550 Mid-Output kit v2.5 (300 cycles) (Illumina Inc). Raw 150 bp paired-end reads were used as input data for the TORMES v.1.2 [39] pipeline for the analysis of whole bacterial genomes. This included sequence quality filtering (PRINSEQ v.0.20.4) [40], *de novo* genome assembly (SPAdes v.13.4.1) [41] and annotation (Prokka v1.14.5) [42]. *E. coli* multilocus sequencing typing (MLST) profiles were predicted using mlst v2.10 (T. Seemann, https://github.com/tseemann/mlst) which uses the PubMLST database [43]. AMR genes were screened using ABRicate (T. Seemann, https://github.com/tseemann/abricate) against the ResFinder [44], Comprehensive Antibiotic Resistance Database (CARD) [45] and ARG-ANNOT [46], databases. Additional software such PointFinder [47] was used to screen for chromosomal point mutations, and PlasmidFinder [48] was used for plasmid replicon screening. Finally, FimTyper [49] and SeroTypeFinder [50] were used to type *E. coli* isolates, whilst *in silico* phylogroup determination was carried out by ClermonTyping [51]. *E. coli* AMR determinants and plasmids were further analyzed using NCBI Blast [52].

A maximum-likelihood phylogenetic tree was constructed to assess *E. coli* clonality. Core genome single nucleotide polymorphisms (SNPs) were identified using CSI phylogeny 1.4 [53]. Reads from each *E. coli* isolate were aligned to a reference genome EC958 (GenBank

accession no. NZ_HG941718.1). EC958 is an international MDR *E. coli* O25b:H4-ST131 clone which produces the CTX-M-15 ESBL and is fluroquinolone resistant [54]. The output file was used to annotate and visualize a phylogenetic tree using the interactive tree of life v.6 (iTOL) tool [55, 56]. Unless otherwise noted, default parameters were used for all software tools.

Metagenomic sequencing and bioinformation analysis

A total of 15 samples were used for shotgun metagenomic sequencing and analysis. Metagenomic sequencing was performed at the SAGC (South Australian Genomics Centre, Adelaide, Australia), and Macrogen Inc. (Seoul, South Korea), using the Illumina Novaseq S1 platform with 150 bp paired ends reads. Read quality was assessed using FastQC [57]. The SqueezeMeta v1.4 [58] pipeline was used for standard metagenomic analysis. SqueezeMeta uses Trimmomatic [59] for filtering and trimming of adapters before assembly using MEGAHIT [60]. The resulting contigs were filtered for quality with short contigs removed with Prinseq [40]. Gene and rRNA predication were performed utilizing the Prodigal gene prediction software [61] while rRNA sequences were found using barrnap [62]. AMR analysis was performed using SqueezeMeta v1.4 [58] by Diamond [63] against version 3.1.4 of the CARD [45]. AMR gene abundance was calculated as transcripts per million (TPM) mapped bacterial reads.

Statistical analysis

Principal components analysis (PCA) was performed in R studio v.1.2.5033 and used here to visually compare the distribution of resistant *E. coli* isolates recovered from wastewater sampled from different sites (facilities). Statistical analysis, bar graphs and boxplots were generated using Graph Prism v9, with statistical differences between selected AMR gene

prevalence and facility assessed by a two-tailed Mann-Whitney U test with results considered statistically significant at $p < 0.05$; $p < 0.01$ level.

Data availability

Whole genome and shotgun metagenomic sequences were deposited in the NCBI database under Bio Project number PRJNA861152. Reads mapping to the human reference genome (GCA_000001405.15) were removed prior to submission to public sequence databases according to the protocol used in the Human Microbiome Project [64, 65].

RESULTS

Resistance profiles of the *E. coli* isolates reveals a high prevalence of resistance and high-level resistance

Wastewater sampling and analysis was used to evaluate the prevalence and antimicrobial resistance of *E. coli* in two local RACFs and one Retirement village. Since fluoroquinolone resistance and ESBL production is of particular concern for *E. coli,* selection of *E. coli* isolates was carried out with selective and differential media supplemented with ciprofloxacin or ceftazidime.

A total of 93 AMR *E. coli* isolates (n=58 from Facility 1, n=27 from Facility 2 and n=8 from a Retirement Village) were purified from wastewater samples. As expected, due to the selection process used in this study, resistance to ceftazidime and ciprofloxacin was observed for a large percentage of *E. coli* isolates, with 66.7 % (n=62/93) and 96.8 % (n=90/93) of all isolates shown to be ceftazidime and ciprofloxacin resistant respectively (Table 1). Interestingly, almost all the isolates were resistant to ciprofloxacin irrespective the media and antibiotic selection/non-selection used for isolation. Also, a high incidence of trimethoprim–

sulfamethoxazole (n=51/93, 54.8 %) and gentamicin (n=47/93, 50.5 %) resistant *E. coli* was also observed although resistance to these antibiotics were not selected for. Finally, resistance to the last resort antibiotics meropenem (n=12/9 3, 20.7 %) and colistin (n=4/93, 4.3 %) was observed, albeit at lower frequencies.

Table 1. MIC distribution for 93 *E. coli* isolates recovered from wastewater sampled from associated RACFs and a retirement village.

	Number of *E. coli* isolates with MIC (mg/L) at:															
Antibiotic	**0.008**	**0.016**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**	**%NWT**
FEP	0	0	3	19	16	5	3	10	6	1	4	6	8	1	11	54
CAZ	0	0	0	0	4	13	14	0	0	1	1	5	18	21	16	67
PTZ	0	0	0	0	0	0	0	9	18	40	10	3	2	7	4	17
CIP	0	0	1	2	0	6	2	8	9	1	5	1	26	18	14	97
GEN	0	0	0	0	0	0	7	22	17	10	2	6	2	6	21	51
MER	8	15	39	19	2	2	5	2	1	0	0	0	0	0	0	13
SXT	1	1	16	11	7	4	2	0	7	0	1	43*	0	0	0	55
COL	0	0	0	0	0	0	13	50	26	0	0	4	0	0	0	4

* MIC of 43 isolates >8 SXT

Vertical black lines indicate EUCAST ECOFF values. FEP, cefepime; CAZ, ceftazidime; PTZ, piperacillin-tazobactam; CIP, ciprofloxacin; GEN, gentamicin; MER, meropenem; SXT, trimethoprim-sulfamethoxazole; COL, colistin; NWT, non-wild-type

Analysis of resistance profiles between facilities reveal a difference in the proportion of multidrug resistant *E. coli* isolates

Analysis of resistance was determined using percentages representing the relative proportion of isolates characteristic of each facility. This was carried out to mitigate the limitation associated with each facility housing a different number of residents. Analysis revealed that out of the 58 isolates from Facility 1 that were assessed, 49 (84.5 %) were found to be non-susceptible to the fourth-generation cephalosporin, cefepime and 57 (98.3 %) were non-susceptible to the third-generation cephalosporin ceftazidime These results contrast with those obtained for Facility 2, with only two out of the 27 (7.4 %) *E. coli* isolates displaying

low to moderate levels of resistance against both cefepime (MICs of up to 2 mg/L) and ceftazidime (MICs of up to 16 mg/L) (Figure 1a and Table 1).

MIC assays revealed high-level ciprofloxacin resistance of ≥ 64 mg/L (more than 1024 times the ECOFF value) for 34.4 % (n=32/93) of all isolates recovered (Table 1). These isolates were relatively equally spread between the two facilities (43.1 % and 25.9 % of the total isolates from Facility 1 and 2 respectively). High-level resistance was also observed against cefepime and ceftazidime with MICs of ≥ 64 mg/L seen in 12.9 % (n=12/93) and 39.8 % (n=37/93) of all isolates for cefepime and ceftazidime respectively. High-level resistance against trimethoprim-sulfamethoxazole (>8 mg/L) was observed for 44 of the 93 isolates (Table 1). Almost all of those isolates (41/58) were from Facility 1 (Figure 1a).

Facility 1 harboured a greater number of *E. coli* isolates resistant to multiple antibiotics (Figure 1b). Facility 2 and Retirement *E. coli* isolates were observed to be primarily gentamicin and ciprofloxacin resistant. Although all isolates assessed in this study were resistant to at least one antibiotic, the MDR phenotype was primarily observed in *E. coli* isolates recovered from Facility 1 (Figure 1b and c), with 54/58 (93.1 %) of the these shown to be resistant to 3 or more antimicrobial classes tested in this study (Figure 1b and c). A much lower frequency (n=5/27, 18.5 %) of MDR *E. coli* were recovered in Facility 2, which was observed to harbour mostly non-MDR *E. coli* isolates.

The largest disparity observed between the isolates assessed in this study pertained to gentamicin resistance, with high levels of resistance (MICs of 64-128 mg/L) observed in 66.7 % (n=18/27) of Facility 2 *E. coli* isolates, and only 8.6 % (n=5/58) of Facility 1 isolates. These findings indicate that initial selection using ciprofloxacin and ceftazidime did not limit the overall results obtained in this study, and that the prevalence of resistant isolates was not determined by the number of occupants per facility. Finally, a low incidence of resistance

was observed for both meropenem and colistin and was observed in isolates recovered from Facility 1 only (Figure 1a).

To further explore the distribution and association between MDR *E. coli* and isolation site, a PCA biplot was constructed (Figure 2). As can be seen, the nature and direction of correlation between resistant isolates and facility indicate that Facility 1 clusters with isolates resistant to numerous antibiotics – as such displaying an MDR phenotype, whilst Facility 2 clustered with Retirement isolates displaying resistance to gentamicin and ciprofloxacin only. Unlike isolates recovered from the two RACFs, which spanned out across the two components, Retirement isolates clusters tightly and were grouped within the Facility 2 cluster. This clustering was expected as the resistance profiles for Facility 2 and Retirement isolates were comparable.

Genomic analysis and isolate typing of a subset of AMR *E. coli*

Of the 93 wastewater *E. coli* isolates analysed in this study, 35 were selected for WGS. Selection was based on their antimicrobial resistance profile and time of sample collection (Table S1). These were selected to further investigate genetic diversity and clonality of the isolates over the sampling period. As a greater number of MDR *E. coli* were isolated from Facility 1, these (n=24/35, 68.6 %) made up most of the samples sequenced. The remaining sequences included seven (20 %) from Facility 2 and four (11.4 %) from Retirement samples.

Genotyping and MLST analysis were used here to investigate the genetic diversity of the isolates sampled in this study. Results revealed 10 different sequence types (ST) and one unknown sequence type among the 35 sequenced *E. coli* isolates (Figure 3). The pandemic ST131 was observed in samples isolated from Facility 1 and represented the largest ST (n=9/24, 37.5 %) of the isolates recovered from this facility. Moreover, two carbapenem

328 resistant *E. coli* isolates that belong to the international high-risk clone ST410 were detected
329 in Facility 1. Facility 2 was represented by ST1286 (n=4/7, 57.1 %), which was also observed
330 in Retirement isolates (n=2/4, 50 %). This sequence type is predominantly associated with
331 environmental and animal isolates [66, 67] and according to the EnteroBase database
332 (https://enterobase.warwick.ac.uk/) has not been isolated previously in Australia. We
333 observed two *E. coli* isolates of emerging pandemic clone ST1193 in Facility 2. This
334 sequence type is the most predominant non-ST131 fluoroquinolone resistant ST in the world
335 [68].

336

337 To further type the sequenced isolates, phylogroup analysis was carried out based on the
338 Clermont phylogroup typing method [51] (Figure 4). Of the seven main phylogroups, A, B1,
339 B2, C, D, E and F [69], 37.1 % (n=13/35) of the sequenced *E. coli* isolates were classified to
340 the increasingly MDR phylogroup B2. A higher prevalence (n=9/35, 25.7 %) of these isolates
341 originated from Facility 1. Phylogroup A, representing commensal strains, was the second
342 most prevalent phylogroup and observed with equal frequencies in Facility 1 and 2 (both
343 n=5/35, 14.3 %). Meanwhile, Retirement *E. coli* isolates were classified in to phylogroups A
344 and B only, mirroring Facility 2 isolates. Of the three different sampling sites, Facility 1
345 isolates represented the greatest diversity in terms of phylogroups, with groups C, D and F
346 also assigned to these isolates. Strains assigned to group C are closely related to phylogroup
347 B1 which represent commensal strains [70]. Phylogroup D isolates, like those classified to
348 group B2 are associated with virulent extra-intestinal *E. coli* infections [71]. Strains assigned
349 to phylogroup F have been found to be ESBL-producing and resistant to fluoroquinolones
350 [72].

351 Finally, core genome SNP analysis was used to assess clonal relatedness of all 35 sequenced
352 *E. coli* isolates, with the results represented in a maximum likelihood phylogenetic tree

(Figure 5). Isolates were aligned to *E. coli* EC958 (accession number: NZ_HG941718.1), which represents an international MDR strain. Analysis revealed a clustering of isolates by facility, sequence type, susceptibility profile and phylogroup indicating clonality. Nonetheless, variability between AMR determinants and plasmid replicons was also observed between clonally related isolates.

Identification of *E. coli* AMR genes

Given the observations that the wastewater isolates assessed in this study displayed a similar resistance profile per facility, we sought to evaluate their genotypic diversity and examined their genomes for the presence of mobile AMR genes and potential plasmids. All these wastewater isolates were shown to possess a plethora of AMR genes, contributing both to their intrinsic and acquired resistance. Twenty-seven different AMR genes conferring resistance to the compounds assessed in this study, which included cephalosporins, carbapenems, β-lactam- β-lactam- inhibitor combinations, fluoroquinolones, aminoglycosides, trimethoprim, and sulphonamides, were identified (Figure 5). The extended spectrum beta-lactamases (ESBLs) such as the $bla_{\text{CTX-M}}$ type genes accounted for 37.3 % (n=19/51) of the beta-lactamase genes detected in the wastewater samples. The $bla_{\text{CTX-M}}$ type genes (n=19) included $bla_{\text{CTX-M-15}}$ (n=4/19, 21.1%), $bla_{\text{CTX-M-27}}$ (n=9/19, 47.4 %) and $bla_{\text{CTX-M-62}}$ (n=6/19, 31.6 %). Except for one $bla_{\text{CTX-M-62}}$ carrying strain isolated from Facility 2, all other $bla_{\text{CTX-M}}$ type genes were found in Facility 1 isolates. Non- ESBL beta-lactamases, $bla_{\text{OXA-1}}$ (n=4/35, 11.4 %) and $bla_{\text{OXA-181}}$ (n= 2/35, 5.7 %) were identified in isolates recovered from Facility 1 only, whereas $bla_{\text{TEM-1}}$ was detected in *E. coli* isolated from Facility 1 (n=8/35, 22.9 %), Facility 2 (n=5/35, 14.3 %) and retirement (n=2/35, 5.7 %). No carbapenemase genes were detected despite meropenem resistance in 4 out of the 6 sequenced meropenem resistant *E. coli* isolates (Figure 5). However, these isolates harboured at least one ESBL or AmpC beta-lactamase genes. Non-carbapenemase-producing low level carbapenem-resistance in *E. coli* could be mediated by ESBL or AmpC beta-lactamases associated with an overexpression of efflux pumps (such as AcrAB) or a loss of porin (OmpF) expression [73, 74].

Bacteria with ESBL phenotypes are frequently found to harbour additional resistance genes. In this study, isolates were found to carry the plasmid-mediated quinolone resistance

(PMQR) genes, which were detected in Facility 1 isolated strains only (n=14/35, 40 %). Of these, the most prevalent PMQR gene detected was *qnrB*4 (n=6/14, 42.9 %). Also only isolated from Facility 1 was the ciprofloxacin and aminoglycoside modifying enzyme *(aac-(6')-lb-cr),* accounting for 14.3 % (n=5/35) of the total isolates detected. Genes mediating aminoglycoside resistance such as *aac(3)-IId* were also detected in 7 out of 35 isolates (20 %) and exclusively recovered from Facility 2 and Retirement, whereas *aph3Ib* and *aph6Id* (both n=13/35, 37.1 %), which are commonly found on plasmids, integrative elements and chromosomal islands, were detected in wastewater isolates recovered from Facility 1. A higher prevalence of the sulfonamide genes, *sul1* (n=21/35, 60 %) and *sul2* (n=15/35, 42.9 %) and trimethoprim *dfrA17* (n=15/35, 42.9 %) resistance genes were also observed, with the majority detected in Facility 1 isolates. Lastly, ciprofloxacin resistance due to point mutations was also investigated, with 32/35 (91.4 %) isolates shown to be phenotypically ciprofloxacin resistant. Of these, 31/32 (96.9 %) were shown to possess mutations within *gyrA, parC* or *parE* genes (Table S2).

Prevalence of plasmid replicon types detected in AMR *E. coli* isolates

Plasmids are known to carry and distribute AMR genes such as ESBLs, carbapenemases, and PMQR genes [75]. Due to their ability to transmit AMR genes to other bacteria, they can enable the rapid evolution of bacteria in response to different environmental pressures [76]. In this study, analysis of plasmids within the 35 sequenced wastewater *E. coli* isolates were differentiated based on plasmid replicon types. The incompatibility groups (Inc) IncFIB (n=22/35, 62.9 %) and IncFII (n=22/35, 62.9 %) accounted for the prevalent replicon types detected in the *E. coli* isolates. All isolates were positive for at least one Inc or replicate (Rep) group. Analysis of the most dominant plasmid replicon type by facility revealed Facility 1 isolates to harbour IncF (IncFIA, IncFIB, IncFIC and IncFII) type plasmids, Facility 2 Col

(ColBS512, ColMG828, ColMP18, Col440I and Col156) type plasmids, with Retirement isolates harbouring both types with near equal distribution.

Relative abundance of bacterial families represented by metagenomics

To compare the complete wastewater resistome with data obtained from the *E. coli* isolates, metagenomics sequences were obtained from all sample sites and used here to assess taxonomy and prevalence of AMR in each site. Taxonomic annotation of the metagenomic datasets revealed the 25 most abundant genera detected in Facility 1, 2 and Retirement wastewater samples (Figure 6). Bacteria belonging to the *Escherichia* genus represented an average of 3.6 % of bacteria in Facility 1, 0.7 % Facility 2 and 0.9 % in Retirement. A comparison between Facility 2 and Retirement revealed a greater number of AMR *E. coli* isolates recovered from Facility 2 despite Retirement presenting an overall greater abundance of these organisms. This exemplifies the importance of culturing in the assessment of prevalence of AMR or MDR organisms within wastewater samples.

Occurrence and abundance of AMR genes in wastewater samples

Given the phenotypic-genotypic relationship in *E. coli* [77-79], this study focused on acquired resistance genes, which, as a result of horizontal gene exchange, have rendered many important pathogens such as MDR *E. coli*, potentially unresponsive to current treatment. The focus of this study included the ESBL $bla_{\text{CTX-M}}$, beta-lactamases bla_{OXA}, bla_{TEM} and bla_{IMP}, the dihydrofolate reductase genes *dfrA1* and the dihydropteroate synthases *sul1* and *sul2*, which confer bacterial resistance against trimethoprim and sulphonamide. In addition to these, PMQR genes *qnrB* and *qnr*S genes and *aac(6')-Ib-cr* gene (cr for

ciprofloxacin resistance) were also evaluated. Finally, prevalence of gentamicin resistance was explored by the assessment of the *aac(3)*- and *aac(6')* – genes.

A higher abundance of most of the acquired resistance genes assessed in this study, by a comparison of TPM values obtained for the AMR genes, were detected in samples originating from Facility 1 (Figure 7a). The exception to this were Facility 2 samples which had a larger proportion of the gentamicin resistance genes *aac(3)*- and *aac(6')*, and Retirement samples which had a higher prevalence of the beta-lactamase bla_{IMP}. The differences between Facility 1 and 2 samples regarding the number of the different AMR genes assessed here were determined to be significant ($p<0.05$; Figure 7b). Differences were seen in beta-lactamase $bla_{\text{CTX-M}}$, bla_{OXA} genes, the dihydrofolate reductase *dfrA1* genes, the 3-N-aminoglycoside acetyltransferase *aac(3)* and the 6′-N-aminoglycoside acetyltransferase *aac(6')* genes, as well as the sulfonamide *sul1* and *sul2* genes and the quinolone antibiotic resistance genes *qnrS*. These results clearly show that Facility 2 has a lower relative abundance of the mobile AMR genes assessed in this study, all of which have been associated with human pathogens. No significant differences were found for the AMR abundance between Facility 2 and Retirement samples.

DISCUSSION

The rise of AMR and emergence of bacteria that are resistant to multiple classes of antibiotics presents a global threat to human health [80]. Measures taken to fight resistance include broadening our understanding of the selective pressures driving AMR to be able to mitigate them. RACFs have been identified as sites harbouring elevated levels of AMR bacteria and presenting an environment which is highly selective to their development and emergence [81, 82].

In this study, the prevalence of AMR *E. coli* was used as an indicator of the incidence and persistence of bacterial resistance in two RACFs, one of which has implemented an AMS program (Facility 2), whilst the other has not (Facility 1). Assessment of the prevalence of *E. coli* in these two RACFs and in one Retirement village was carried out with an in-depth analysis of 93 AMR *E. coli* isolates recovered from wastewater samples, collected over five different time points. Results revealed a high proportion of MDR *E. coli* isolates (66.7 %) which were more frequently recovered from Facility 1. Isolates recovered from Facility 1 displayed high levels of resistance against ceftazidime, cefepime and ciprofloxacin with MIC values of >128, >512 and >1024 times above the ECOFF. While Facility 2 isolates displayed high-level gentamicin resistance, with MIC values of >64 mg/L (>32 times above the ECOFF), a trend not observed in Facility 1 *E. coli* isolates. These results highlight several concerns, including the high prevalence of ESBL producing *E. coli* in Facility 1 and the number of ciprofloxacin resistant *E. coli* isolates prevalent in both facilities.

According to the Antimicrobial Use and Resistance in Australia (AURA) Surveillance System (2019) rates of resistance for many priority organisms, including *E. coli*, have not changed significantly over the last 2 years. However, there are several exceptions to this, including increasing rates of resistance observed in *E. coli* against commonly used agents such as ceftriaxone, a third-generation cephalosporin and ciprofloxacin, amongst other fluoroquinolones, despite restricted access in the community and guidelines advising these not to be prescribed when other options are available, and if needed to be prescribed for the shortest duration clinically possible (AURA, 2019). Of the two cephalosporins assessed here, higher rates (66.7 %) of resistance were seen for the third-generation ceftazidime, than for the fourth-generation extended spectrum agent, cefepime (53.7% of all isolates). Whole genome sequencing of ESBL producing *E. coli* revealed the presence of CTX-M type genes assigned to ST131 isolates. This sequence type is a major contributor to both sepsis and urinary tract

480 infections (UTIs) in Australia [83]. Moreover, phylo-typing assigned these isolates to the B2
481 phylogenetic group. This group is frequently associated with CTX-M-15 producing *E. coli*
482 [84], although in this study was associated with CTX-M-27 producing isolates. Globally,
483 CTX-M-15 producing strains are recognised as the main drivers of the pandemic spread of
484 this sequence type [85]. However, genomic comparisons of ST131 isolates have shown that
485 both CTX-M-15 and CTX-M-27 producing isolates dominate in Australia [83]. CTX-M-27
486 ESBLs were also initially postulated to be confined to ST131 [86], however, these have now
487 also been identified in ST38 *E. coli* isolates, as also observed in this study, suggesting an
488 emergence of new ESBL-producing clones [87]. The dissemination and continual emergence
489 of these ESBLs and others are facilitated by their presence on plasmids belonging to the IncF
490 group [88], amongst others, which were also detected here. This plasmid group is often
491 associated with $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and *aac(6')lb-cr* resistance genes, all of which
492 were detected here and largely in isolates recovered from Facility 1.

493 The isolation of ST131 in this study highlights the continual spread of this pandemic clonal
494 group. Although a worrying trend, the presence of ST131 *E. coli* isolates within RACFs has
495 been observed worldwide [89, 90]. It has been postulated that isolates of this sequence type
496 have superior transmission and colonisation abilities, allowing for rapid global proliferation
497 [91-93]. These characteristics coupled with the intrinsic nature of RACFs, and residents
498 therein makes the presence of this clonal group within RACFs a significant concern.

499 In addition to being resistant to extended spectrum cephalosporins, ST131 *E. coli* clones are
500 also associated with fluroquinolone resistance [94] which can be coupled with
501 aminoglycoside and trimethoprim-sulfamethoxazole resistance [92]. In this study, all ST131
502 isolates were ciprofloxacin resistant and except for one isolate, trimethoprim-
503 sulfamethoxazole resistant. Worryingly, two isolates were extensively drug resistant with
504 resistance against all the tested classes of antibiotics observed. Fluroquinolone resistance

505 continues to be of concern and was observed in this study with high levels of ciprofloxacin
506 resistance (MICs of > 64 mg/L) detected in isolates recovered from both facilities, and in
507 Retirement samples. According to the Australian Group of Antimicrobial Resistance (AGAR)
508 Sepsis Outcome Programs, 2021, rates of fluroquinolone resistant *E. coli* have continued to
509 increase from 13.7 % in 2013 to 21.8 % in 2021. Increasing trends have also been observed
510 in Europe with the European Antimicrobial Resistance Surveillance Network (EARS-Net)
511 reporting 25.3 % fluoroquinolone resistant *E. coli* isolated in 2018, representing an increase
512 from previous years [95]. This upward trend is also reported in *E. coli* isolated in Asia as well
513 as North America [96]. Various theories have been presented regarding the acquisition of
514 fluoroquinolone resistant *E. coli* including possible colonisation with these as a result of
515 hospital admissions [97], the continual use of these in treatment of UTIs [98] and clonal
516 spread [99].

517 Analysis of fluoroquinolone resistance genes within WGS isolates revealed the presence of
518 numerous ciprofloxacin resistance genes including the transferable plasmid-mediated
519 quinolone resistance (PMQR) determinants *qnr* and *aac(6)-Ib-cr.* The presence of these is of
520 concern as these are able to be transferred to other bacteria, are often associated with
521 elements such as insertion sequences, transposons, phages and plasmids which enhances their
522 transferral and can be accompanied by other mobile resistance genes [100].

523 Metagenomic analysis of the prevalence of these and other mobile resistance genes within the
524 sampled wastewater revealed that Facility 1 harboured a greater number of mobile resistance
525 determinants. A limitation of using the metagenomic method for the assessment of bacterial
526 and AMR gene abundance is that each sample of wastewater represents a single point in time.
527 As such, wastewater components may be influenced by the amount of faecal matter in the
528 samples, the activities being performed at the time of collection, such as cleaning or washing
529 and the detergents being used, all of which may alter the microbial and antimicrobial gene

abundance within each sample. To minimise this limitation, sites were sampled over a 10-hour period at five different time points throughout an 18-month period and samples assessed by microbial culturing and metagenomics. To ensure that the data are not influenced by the number of people, flow rate at the timepoints of grab sample collection or any other potential variables, the data obtained from metagenomics was normalised and AMR gene abundance was calculated as transcripts per million mapped bacterial reads. In addition to Facility 1 samples, which displayed consistently higher levels of bacteria belonging to the *Escherichia* genus, metagenomic results also revealed a higher prevalence of these in Retirement wastewater samples when compared to Facility 2, but an overall lower prevalence of AMR *E. coli* upon culturing. As such, the higher prevalence of AMR *E. coli*, as identified by microbial culturing and metagenomics for all five sampling periods, is indicative of a general trend only seen for Facility 1. This trend was also observed with respect to mobile resistance genes, with Facility 1 displaying a comparatively higher abundance of certain resistance genes.

Analysis of the mobile resistance genes revealed plasmid-mediated resistance genes, some of which are associated with Class 1 integrons that have been isolated from AMR clinical isolates belonging to both the Enterobacteriaceae and Pseudomonaceae families [101]. Having the ability to integrate gene cassettes which confer resistance to a broad range of antibiotics has made the prevalence of Class 1 integrons a proxy for bacterial resistance [102]. As such, the greater presence of the mobile resistance genes may be indicative of a higher presence of AMR bacteria in Facility 1 wastewater.

Limitations of this study include that only two RACFs were assessed and compared, and that these differed in the number of residents residing in them. However, all variables that were reported in this study were the same between the facilities, for example the age of the cohort and type of care provided. Moreover, the two facilities were managed by the same age care

555 provider, which runs additional facilities in Australia. It is possible that there could have been
556 differences in variables that were not measured as part of this study such as the socio-
557 economic status of the demographic, duration of stay, staff turnover number or age of the
558 facility. To overcome variations in resident numbers and isolate prevalence, percentages were
559 utilized for standardized comparisons, allowing meaningful assessments across facilities in
560 the study. However, to our knowledge this is the first study to include such a comprehensive,
561 all-encompassing testing protocol. This study has included isolation of *E. coli* and culture
562 based genotypic AMR analysis, genomic analysis of the resistome of individual organisms
563 and metagenomic sequencing and analysis of the total wastewater resistome. It is also the
564 first time that a direct comparison of two RACFs has been undertaken, with a spotlight on
565 AMR.

566 The findings of this study offer an insight into bacterial resistance and the prevalence thereof
567 within RACFs. Here, one facility was found to harbour higher rates of AMR *E. coli,*
568 including the pandemic clone ST131. Moreover, metagenomic analysis revealed a higher
569 incidence of mobile resistance determinants in wastewater sourced from this facility. The
570 inclusion of microbial culturing in combination with whole genome sequencing and
571 metagenomic analysis has allowed for a more complete assessment of the RACFs evaluated
572 in this study. Based on the differences in the prevalence of AMR *E. coli* and mobile
573 resistance genes within the RACFs assessed here, our results show that each facility presents
574 a unique environment which may select for resistance and possible transmission of AMR
575 bacteria. Mitigating this may be the implementation of an AMS program, which was present
576 in Facility 2. However, it is understandable that the implementation of AMS programs in
577 RACFs can be complicated and is dependent on numerous factors including access to
578 resources, staff education and a high prevalence of bacterial colonisation and challenges in

579 diagnosing and treating infections [103]. Nonetheless, the benefits of such programs can help
580 mitigate the spread and continual development of AMR.

581 Currently there are significant gaps in the surveillance of AMR and MDR organisms within
582 RACFs. Our understanding of the colonisation of such organisms in RACF residents is also
583 lacking, as is knowledge pertaining to the risk factors which aid both the development of
584 AMR and potential spread of both AMR and MDR organisms and high-risk antimicrobial
585 genes from RACFs to the wider community. The results generated in this study highlight the
586 need for ongoing surveillance studies focussing on RACFs. Which, given our ageing
587 population, are greatly needed to address and possibly mitigate the ongoing threat of AMR.

588

589 **SUPPLEMENTARY MATERIAL**

590 Supplemental 1. AMS Strategies

591 Table S1. Overview of antibiotic resistance profiles, with MIC and gene profiles (0 = absent
592 or 1 = present) of wastewater *E. coli* isolated from Facility 1 and 2 and from Retirement,
593 Adelaide, Australia.

594 Table S2: GyrA, ParC and ParE mutations in 35 wastewater *E. coli* isolates corresponding
595 ciprofloxacin MICs.

596

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Figure 1: Facility 1 shows the highest prevalence of MDR *E. coli* isolates. **(A)** Percentage of total AMR *E. coli* isolates per facility and retirement village. **(B)** Prevalence of *E. coli* isolates (n=93) per facility and number of antibiotics (total n=8) each isolate was resistant against. **(C)** Number of MDR and non-MDR *E. coli* isolates per facility. Facility 1 n=58, Facility 2 n=27 and Retirement n=8. FEP, cefepime; CAZ, ceftazidime; PIP-TAZ, piperacillin-tazobactam; CIP, ciprofloxacin; GEN, gentamicin; MER, meropenem; SXT, trimethoprim-sulfamethoxazole; COL, colistin, Ab (s), antibiotic(s).

Figure 2: Principal component analysis (PCA) plot shows clustering of MDR *E. coli* isolates recovered from Facility 1 (blue) relative to isolates which are resistant to gentamicin (GEN) and ciprofloxacin (CIP) only and recovered from Facility 2 (red) and Independent Living Facility (Retirement, grey). Ellipses were drawn at a confidence level of 0.95. CEP, cefepime; CTZ, ceftazidime; PIT, piperacillin-tazobactam; CIP, ciprofloxacin; GEN, gentamicin; MER, meropenem; TRS, trimethoprim-sulfamethoxazole; COL, colistin.

Figure 3. Distribution and percentage of sequenced *E. coli* isolate MLSTs per facility and retirement village. Facility 1 n=24, Facility 2 n=7 and Retirement n=4.

Figure 4: Phylogroup distribution of 35 *E. coli* isolates from two RACFs and a Retirement site. Facility 1 n=24, Facility 2 n=7 and Retirement n=4.

Figure 5: Phylogenetic SNP analysis of 35 AMR *E. coli* isolated from RACFs and a Retirement Village in South Australia, corresponding resistance profile presence of AMR determinants and plasmid replicons. SNP analysis was performed using the isolate EC958 (accession number: NZ_HG941718.1) as the reference genome. Vertical columns demonstrate: (1) isolation site, (2) phylogroups (3) resistance phenotype: resistant (filled squares) and sensitive (empty squares) (4) resistance genotype including plasmid mediated quinolone resistance (PMQR): presence (filled circles) and absence (empty circles) of acquired resistance genes (5) mutations in the quinolone resistance-determining regions: present (blue filled rectangle) and absent (empty rectangle) (7) predominant plasmid replicon types: presence (filled left side triangles) and absence (empty left side triangles). FEP, cefepime; CAZ, ceftazidime; PIT, piperacillin-tazobactam; CIP, ciprofloxacin; GEN, gentamicin; MER, meropenem; SXT, trimethoprim-sulfamethoxazole; COL, colistin.

Figure 6. Relative abundance (%) of the top 25 genera identified in wastewater collected from two RACFs and Retirement samples. Red bars represent bacteria belonging to the *Escherichia* genus.

Figure 7: Distribution of mobile resistance genes in wastewater from RACFs and an independent living facility (Retirement). **(A)** Heatmap of distribution and abundance clustering of resistance genes. AMR genes are shown on the right vertical axis. Hierarchical clustering was carried out on both resistance genes and sites. AMR abundance is normalised as TMP (transcripts per million reads). **(B)** Boxplots of select AMR gene reads per ten million (TPM) per facility. Each sample is represented by a dot with horizontal jitter for visibility. The horizontal box lines represent the first quartile, the median, and the third quartile. Whiskers represent the range of points within the first quartile − 1.5× the interquartile range and the third quartile + 1.5× the interquartile range. Differences were considered statistically significant at * $p < 0.05$; **$p < 0.01$ level, determined by a two-tailed Mann-Whitney U test.

References

1. O'Neill, J., *Tackling a crisis for the health and wealth of nations.* Antimicrobial Resistance, 2014.
2. Murray, C.J., et al., *Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis.* The Lancet, 2022.
3. WHO, *Antimicrobial resistance: Global report on surveillance* Bull. World Health Organ, 2014. **62**: p. 383-394.
4. Prestinaci, F., P. Pezzotti, and A. Pantosti, *Antimicrobial resistance: a global multifaceted phenomenon.* Pathogens and global health, 2015. **109**(7): p. 309-318.
5. Mouton, C.P., et al., *Common infections in older adults.* American family physician, 2001. **63**(2): p. 257.
6. Gavazzi, G. and K.-H. Krause, *Ageing and infection.* The Lancet Infectious Diseases, 2002. **2**(11): p. 659-666.
7. Kline, K.A. and D.M.E. Bowdish, *Infection in an aging population.* Current Opinion in Microbiology, 2016. **29**: p. 63-67.

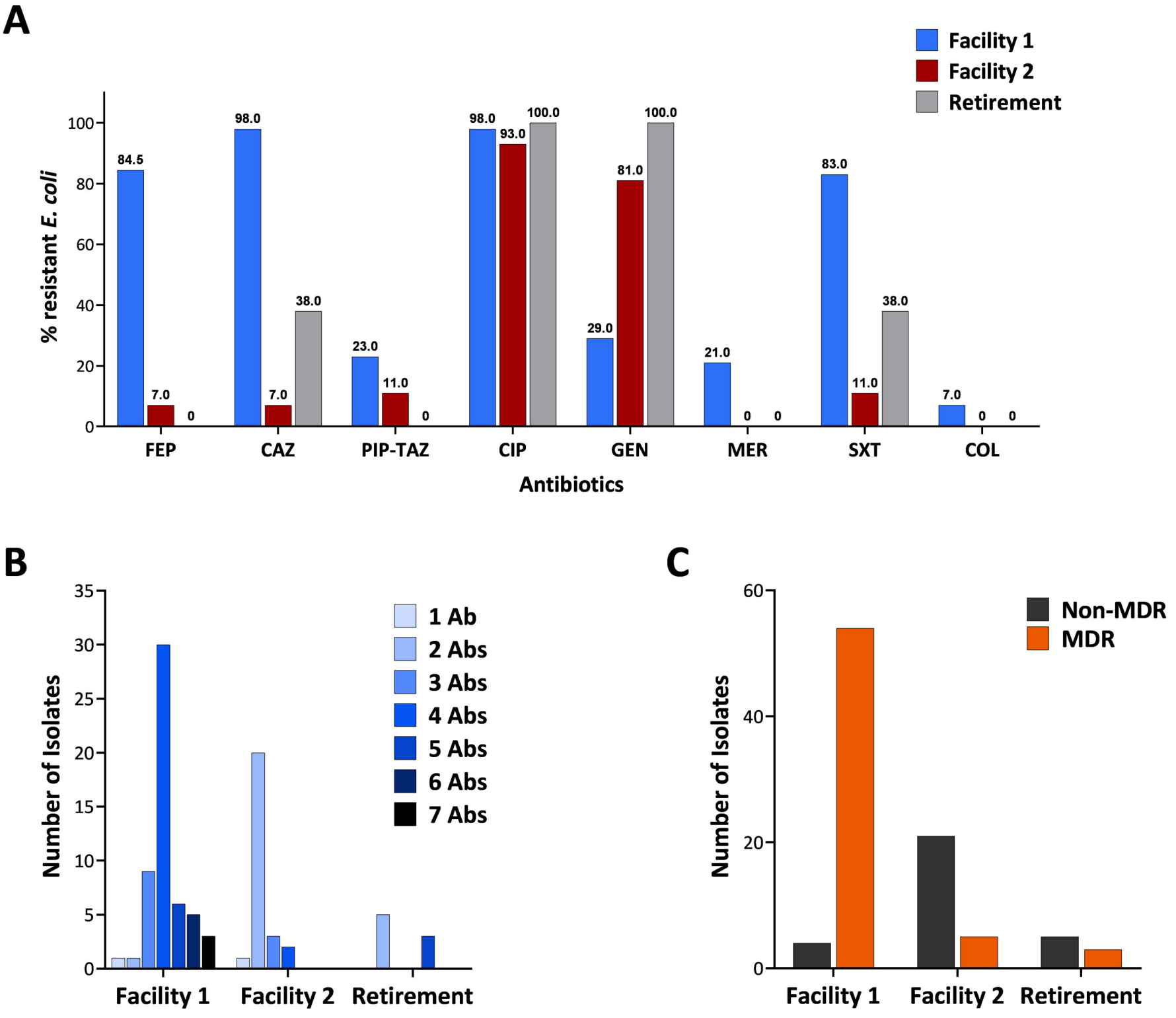
8. Bell, B.G., et al., *A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance.* BMC infectious diseases, 2014. **14**(1): p. 1-25.
9. Costelloe, C., et al., *Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis.* Bmj, 2010. **340**.
10. Wojkowska-Mach, J., et al., *Antibiotic consumption and antimicrobial resistance in Poland; findings and implications.* Antimicrobial Resistance & Infection Control, 2018. **7**(1): p. 1-10.
11. Llor, C. and L. Bjerrum, *Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem.* Therapeutic Advances in Drug Safety, 2014. **5**(6): p. 229-241.
12. Sluggett, J.K., et al., *National trends in antibiotic use in Australian Residential Aged Care Facilities, 2005-2016.* Clinical Infectious Diseases, 2020: p. 1-8.
13. Fleming, A., et al., *Antibiotic prescribing in long-term care facilities: a meta-synthesis of qualitative research.* Drugs Aging, 2015. **32**(4): p. 295-303.
14. Thornley, T., et al., *Antibiotic prescribing for residents in long-term-care facilities across the UK.* J Antimicrob Chemother, 2019. **74**(5): p. 1447-1451.
15. Yates, M., et al., *A study of infection in elderly nursing/residential home and community-based residents.* J Hosp Infect, 1999. **43**(2): p. 123-9.
16. Ingarfield, S.L., et al., *Use of emergency departments by older people from residential care: a population based study.* Age Ageing, 2009. **38**(3): p. 314-8.
17. O'Fallon, E., et al., *Acquisition of multidrug-resistant gram-negative bacteria: incidence and risk factors within a long-term care population.* Infect Control Hosp Epidemiol, 2010. **31**(11): p. 1148-53.
18. Lim, C.J., R.L. Stuart, and D.C. Kong, *Antibiotic use in residential aged care facilities.* Australian Family Physician, 2015. **44**(4): p. 192-6.
19. Zignol, M., et al., *Twenty years of global surveillance of antituberculosis-drug resistance.* New England Journal of Medicine, 2016. **375**(11): p. 1081-1089.
20. Weston, E., T. Wi, and J. Papp, *Strengthening Global Surveillance for Antimicrobial Drug–Resistant Neisseria gonorrhoeae through the Enhanced Gonococcal Antimicrobial Surveillance Program.* Emerging Infectious Disease journal, 2017. **23**(13).
21. Bäumlisberger, M., et al., *Influence of a non-hospital medical care facility on antimicrobial resistance in wastewater.* PLoS One, 2015. **10**(3): p. e0122635.
22. Stuart, R.L., et al., *Prevalence of antimicrobial-resistant organisms in residential aged care facilities.* Medical Journal of Australia, 2011. **195**(9): p. 530-533.
23. Ludden, C., et al., *Colonisation with ESBL-producing and carbapenemase-producing Enterobacteriaceae, vancomycin-resistant enterococci, and meticillin-resistant Staphylococcus aureus in a long-term care facility over one year.* BMC Infect Dis, 2015. **15**: p. 168.
24. Valenza, G., et al., *Prevalence and genetic diversity of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli in nursing homes in Bavaria, Germany.* Veterinary Microbiology, 2017. **200**: p. 138-141.
25. Arvand, M., V. Moser, and Y. Pfeifer, *Prevalence of extended-spectrum-β-lactamase-producing Escherichia coli and spread of the epidemic clonal lineage ST131 in nursing homes in Hesse, Germany.* Journal of Antimicrobial Chemotherapy, 2013. **68**(11): p. 2686-2688.
26. Luvsansharav, U.-O., et al., *Fecal carriage of CTX-M β-lactamase-producing Enterobacteriaceae in nursing homes in the Kinki region of Japan.* Infection and drug resistance, 2013. **6**: p. 67-70.
27. Overdevest, I., et al., *Prolonged colonisation with Escherichia coli O25:ST131 versus other extended-spectrum beta-lactamase-producing E. coli in a long-term care facility with high endemic level of rectal colonisation, the Netherlands, 2013 to 2014.* Euro Surveill, 2016. **21**(42).
28. Rodrigues, C., et al., *Different Escherichia coli B2-ST131 clades (B and C) producing extended-spectrum β-lactamases (ESBL) colonizing residents of Portuguese nursing homes.* Epidemiology and Infection, 2017. **145**(15): p. 3303-3306.

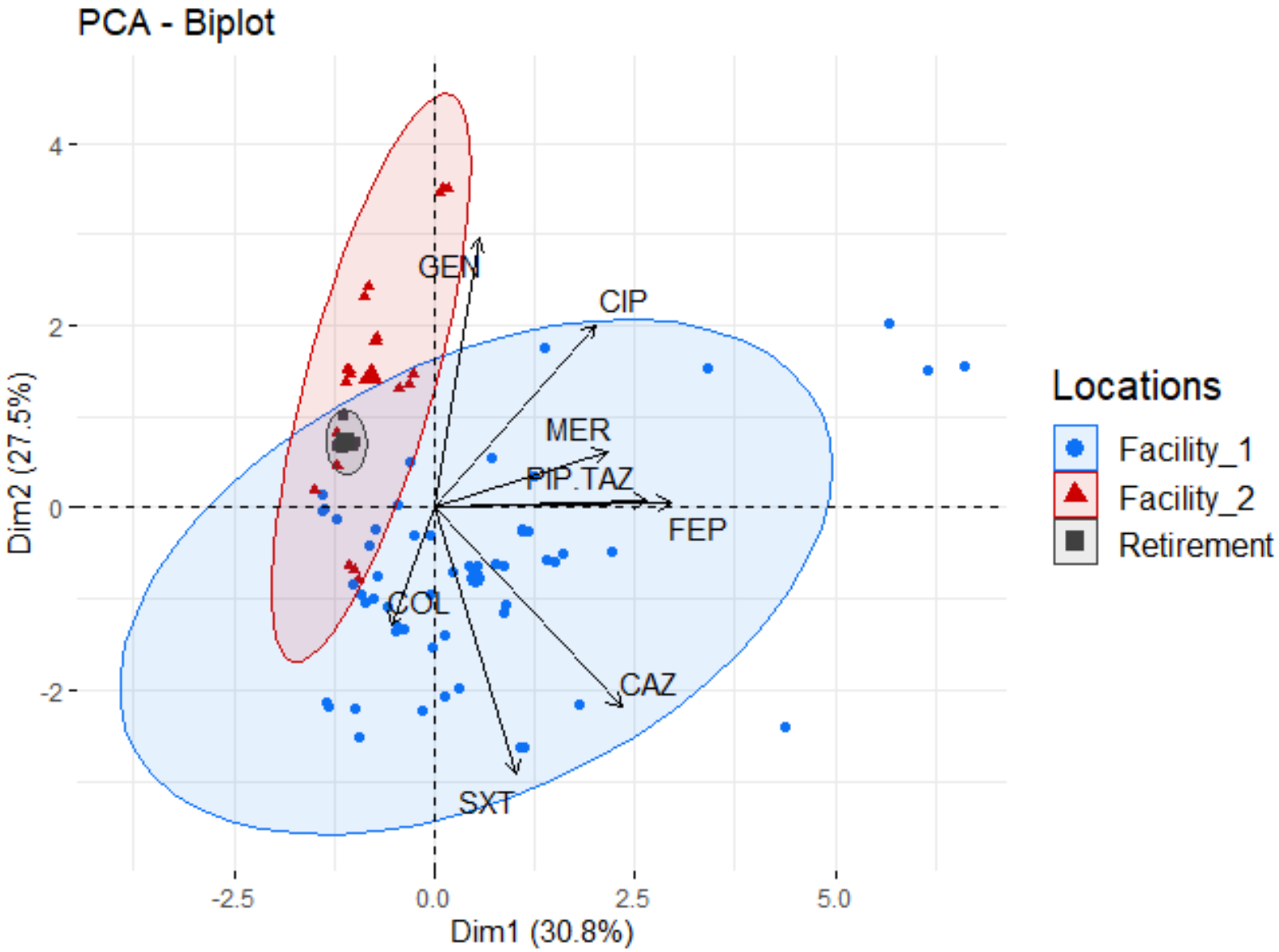
29. Rogers, B.A., H.E. Sidjabat, and D.L. Paterson, *Escherichia coli O25b-ST131: a pandemic, multiresistant, community-associated strain.* J Antimicrob Chemother, 2011. **66**(1): p. 1-14.
30. Nicolas-Chanoine, M.H., et al., *Intercontinental emergence of Escherichia coli clone O25:H4-ST131 producing CTX-M-15.* J Antimicrob Chemother, 2008. **61**(2): p. 273-81.
31. Jones, G.L., et al., *Prevalence and distribution of plasmid-mediated quinolone resistance genes in clinical isolates of Escherichia coli lacking extended-spectrum β-lactamases.* Journal of antimicrobial chemotherapy, 2008. **62**(6): p. 1245-1251.
32. ACSQHC, A.C.o.S.Q.i.H.C., *AURA 2021: Fourth Australian Report on Antimicrobial Use and Resistance in Human Health*. 2021, ACSQHC Sydney, Australia.
33. WHO. *Ageing and health* 2022; Available from: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health.
34. International Organization for Standardization, *Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. ISO, Switzerland: Geneva.* 2019.
35. Amsalu, A., et al., *Worldwide distribution and environmental origin of the Adelaide imipenemase (AIM-1), a potent carbapenemase in Pseudomonas aeruginosa.* Microbial Genomics, 2021. **7**(12).
36. Chai, W.C., et al., *Cinnamaldehyde derivatives act as antimicrobial agents against Acinetobacter baumannii through the inhibition of cell division.* Frontiers in Microbiology, 2022. **13**.
37. Magiorakos, A.-P., et al., *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.* Clinical microbiology and infection, 2012. **18**(3): p. 268-281.
38. Amsalu, A., et al., *Efflux pump-driven antibiotic and biocide cross-resistance in Pseudomonas aeruginosa isolated from different ecological niches: a case study in the development of multidrug resistance in environmental hotspots.* Microorganisms, 2020. **8**(11): p. 1647.
39. Quijada, N.M., et al., *TORMES: an automated pipeline for whole bacterial genome analysis.* Bioinformatics, 2019. **35**(21): p. 4207-4212.
40. Schmieder, R. and R. Edwards, *Quality control and preprocessing of metagenomic datasets.* Bioinformatics, 2011. **27**(6): p. 863-864.
41. Bankevich, A., et al., *SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing.* Journal of Computational Biology, 2012. **19**(5): p. 455-477.
42. Seemann, T., *Prokka: rapid prokaryotic genome annotation.* Bioinformatics, 2014. **30**(14): p. 2068-2069.
43. Jolley, K.A. and M.C.J. Maiden, *BIGSdb: Scalable analysis of bacterial genome variation at the population level.* BMC Bioinformatics, 2010. **11**(1): p. 595.
44. Zankari, E., et al., *Identification of acquired antimicrobial resistance genes.* Journal of antimicrobial chemotherapy, 2012. **67**(11): p. 2640-2644.
45. McArthur, A.G., et al., *The comprehensive antibiotic resistance database.* Antimicrobial agents and chemotherapy, 2013. **57**(7): p. 3348-3357.
46. Gupta, S.K., et al., *ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes.* Antimicrobial agents and chemotherapy, 2014. **58**(1): p. 212-220.
47. Zankari, E., et al., *PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens.* Journal of Antimicrobial Chemotherapy, 2017. **72**(10): p. 2764-2768.
48. Carattoli, A., et al., *In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing.* Antimicrobial agents and chemotherapy, 2014. **58**(7): p. 3895-3903.
49. Roer, L., et al., *Development of a Web Tool for Escherichia coli Subtyping Based on fimH Alleles.* J Clin Microbiol, 2017. **55**(8): p. 2538-2543.

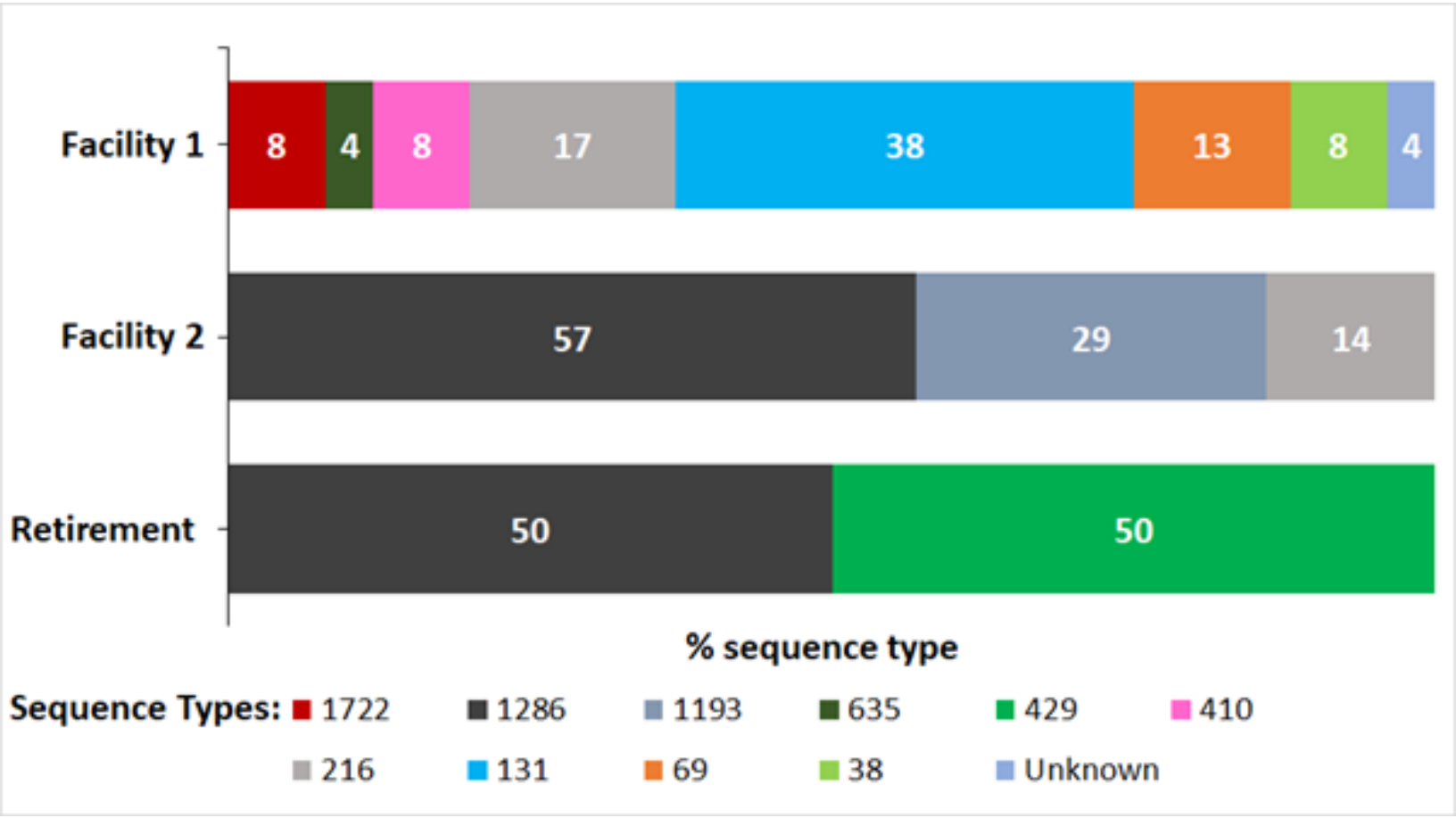
50. Joensen, K.G., et al., *Rapid and Easy In Silico Serotyping of Escherichia coli Isolates by Use of Whole-Genome Sequencing Data.* Journal of Clinical Microbiology, 2015. **53**(8): p. 2410-2426.
51. Beghain, J., et al., *ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping.* Microbial genomics, 2018. **4**(7): p. e000192.
52. Altschul, S.F., et al., *Basic local alignment search tool.* Journal of molecular biology, 1990. **215**(3): p. 403-410.
53. Kaas, R.S., et al., *Solving the Problem of Comparing Whole Bacterial Genomes across Different Sequencing Platforms.* PLOS ONE, 2014. **9**(8): p. e104984.
54. Forde, B.M., et al., *The complete genome sequence of Escherichia coli EC958: a high quality reference sequence for the globally disseminated multidrug resistant E. coli O25b:H4-ST131 clone.* PLoS One, 2014. **9**(8): p. e104400.
55. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation.* Bioinformatics, 2007. **23**(1): p. 127-8.
56. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation.* Nucleic Acids Research, 2021. **49**(W1): p. W293-W296.
57. Andrews, S., *FastQC: A Quality Control Tool for High Throughput Sequence Data [Online].* 2010.
58. Tamames, J. and F. Puente-Sánchez, *SqueezeMeta, A Highly Portable, Fully Automatic Metagenomic Analysis Pipeline.* Frontiers in Microbiology, 2019. **9**(3349).
59. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics (Oxford, England), 2014. **30**(15): p. 2114-2120.
60. Li, D., et al., *MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.* Bioinformatics, 2015. **31**(10): p. 1674-1676.
61. Hyatt, D., et al., *Prodigal: prokaryotic gene recognition and translation initiation site identification.* BMC Bioinformatics, 2010. **11**(1): p. 119.
62. Seemann, T., *Barrnap 0.7: rapid ribosomal RNA prediction.* 2013.
63. Buchfink, B., K. Reuter, and H.-G. Drost, *Sensitive protein alignments at tree-of-life scale using DIAMOND.* Nature methods, 2021. **18**(4): p. 366-368.
64. Sherry, S., *Human sequence removal.* National Center or Biotechnology Information. Human Microbiome Project, 2011.
65. Human Microbiome Project, *GRCh38p12* https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.38/. 2020.
66. Dominguez, J.E., et al., *Characterization of Escherichia coli Carrying mcr-1-Plasmids Recovered From Food Animals From Argentina.* Frontiers in Cellular and Infection Microbiology, 2019. **9**.
67. Wu, C., et al., *Rapid rise of the ESBL and mcr-1 genes in Escherichia coli of chicken origin in China, 2008–2014.* Emerging Microbes & Infections, 2018. **7**(1): p. 1-10.
68. Pitout, J.D., et al., *Escherichia coli ST1193: Following in the Footsteps of E. coli ST131.* Antimicrobial Agents and Chemotherapy, 2022: p. e00511-22.
69. Clermont, O., et al., *The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups.* Environmental microbiology reports, 2013. **5**(1): p. 58-65.
70. Moissenet, D., et al., *Meningitis caused by Escherichia coli producing TEM-52 extended-spectrum β-lactamase within an extensive outbreak in a neonatal ward: epidemiological investigation and characterization of the strain.* Journal of clinical microbiology, 2010. **48**(7): p. 2459-2463.
71. Clermont, O., D. Gordon, and E. Denamur, *Guide to the various phylogenetic classification schemes for Escherichia coli and the correspondence among schemes.* Microbiology, 2015. **161**(5): p. 980-988.
72. Vangchhia, B., et al., *Phylogenetic diversity, antimicrobial susceptibility and virulence characteristics of phylogroup F Escherichia coli in Australia.* Microbiology (Reading), 2016. **162**(11): p. 1904-1912.

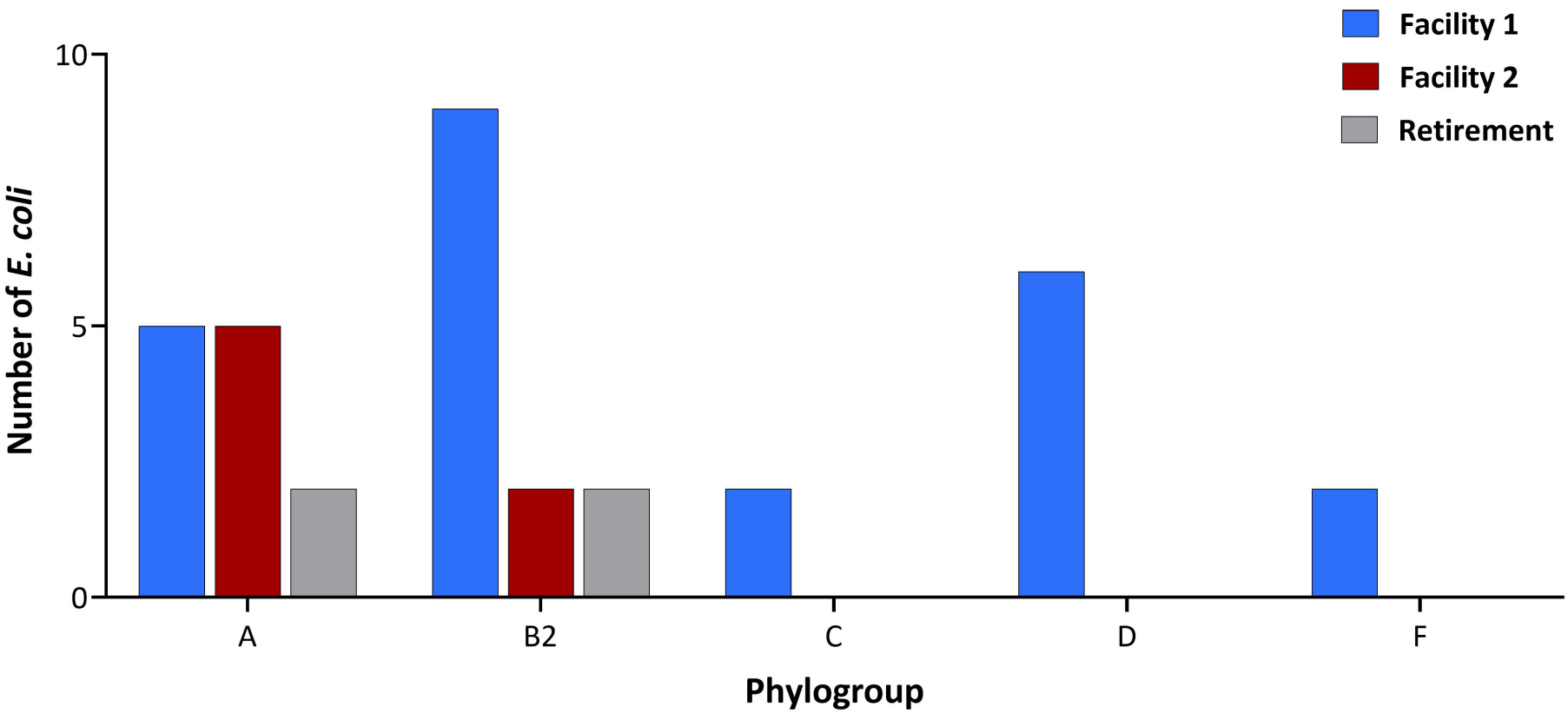
73. Chetri, S., et al., *AcrAB-TolC efflux pump system plays a role in carbapenem non-susceptibility in Escherichia coli.* BMC microbiology, 2019. **19**(1): p. 1-7.
74. Yang, W.-T., et al., *Comparative Genomics Revealed Fluoroquinolone Resistance Determinants and OmpF Deletion in Carbapenem-Resistant Escherichia coli.* Frontiers in microbiology, 2022. **13**.
75. Carattoli, A., *Plasmids and the spread of resistance.* Int J Med Microbiol, 2013. **303**(6-7): p. 298-304.
76. Orlek, A., et al., *Plasmid Classification in an Era of Whole-Genome Sequencing: Application in Studies of Antibiotic Resistance Epidemiology.* Frontiers in Microbiology, 2017. **8**.
77. Ruppé, E., et al., *From genotype to antibiotic susceptibility phenotype in the order Enterobacterales: a clinical perspective.* Clinical Microbiology and Infection, 2020. **26**(5): p. 643. e1-643. e7.
78. Tyson, G.H., et al., *WGS accurately predicts antimicrobial resistance in Escherichia coli.* Journal of Antimicrobial Chemotherapy, 2015. **70**(10): p. 2763-2769.
79. Ellington, M., et al., *The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee.* Clinical microbiology and infection, 2017. **23**(1): p. 2-22.
80. Venter, H., M.L. Henningsen, and S.L. Begg, *Antimicrobial resistance in healthcare, agriculture and the environment: the biochemistry behind the headlines.* Essays in biochemistry, 2017. **61**(1): p. 1-10.
81. Stuart, R.L., et al., *Antibiotic use and misuse in residential aged care facilities.* Internal Medicine Journal, 2012. **42**(10): p. 1145-1149.
82. Weekes, L.M., *Antibiotic resistance changing management of urinary tract infections in aged care.* Medical Journal of Australia, 2015. **203**(9): p. 352-352.
83. Li, D., et al., *Genomic comparisons of Escherichia coli ST131 from Australia.* Microbial genomics, 2021. **7**(12).
84. Coque, T.M., et al., *Dissemination of clonally related Escherichia coli strains expressing extended-spectrum beta-lactamase CTX-M-15.* Emerg Infect Dis, 2008. **14**(2): p. 195-200.
85. Nicolas-Chanoine, M.H., X. Bertrand, and J.Y. Madec, *Escherichia coli ST131, an intriguing clonal group.* Clin Microbiol Rev, 2014. **27**(3): p. 543-74.
86. Matsumura, Y., et al., *Association of fluoroquinolone resistance, virulence genes, and IncF plasmids with extended-spectrum-β-lactamase-producing Escherichia coli sequence type 131 (ST131) and ST405 clonal groups.* Antimicrobial agents and chemotherapy, 2013. **57**(10): p. 4736-4742.
87. Cameron, A., et al., *Detection of CTX-M-27 β-lactamase genes on two distinct plasmid types in ST38 Escherichia coli from three US states.* Antimicrobial agents and chemotherapy, 2021. **65**(7): p. e00825-21.
88. Carattoli, A., *Resistance plasmid families in Enterobacteriaceae.* Antimicrobial agents and chemotherapy, 2009. **53**(6): p. 2227-2238.
89. Aschbacher, R., et al., *Review on colonization of residents and staff in Italian long-term care facilities by multidrug-resistant bacteria compared with other European countries.* Antimicrobial Resistance & Infection Control, 2016. **5**(1): p. 1-9.
90. Burgess, M.J., et al., *Long-Term Care Facilities Are Reservoirs for Antimicrobial-Resistant Sequence Type 131 Escherichia coli.* Open forum infectious diseases, 2015. **2**(1): p. ofv011-ofv011.
91. Banerjee, R. and J.R. Johnson, *A new clone sweeps clean: the enigmatic emergence of Escherichia coli sequence type 131.* Antimicrobial agents and chemotherapy, 2014. **58**(9): p. 4997-5004.
92. Banerjee, R., et al., *Escherichia coli sequence type 131 is a dominant, antimicrobial-resistant clonal group associated with healthcare and elderly hosts.* Infection control and hospital epidemiology, 2013. **34**(4): p. 361-369.
93. Ben Zakour, N.L., et al., *Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of Escherichia coli ST131.* MBio, 2016. **7**(2): p. e00347-16.
94. Schembri, M.A., et al., *Molecular Characterization of the Multidrug Resistant Escherichia coli ST131 Clone.* Pathogens, 2015. **4**(3): p. 422-430.

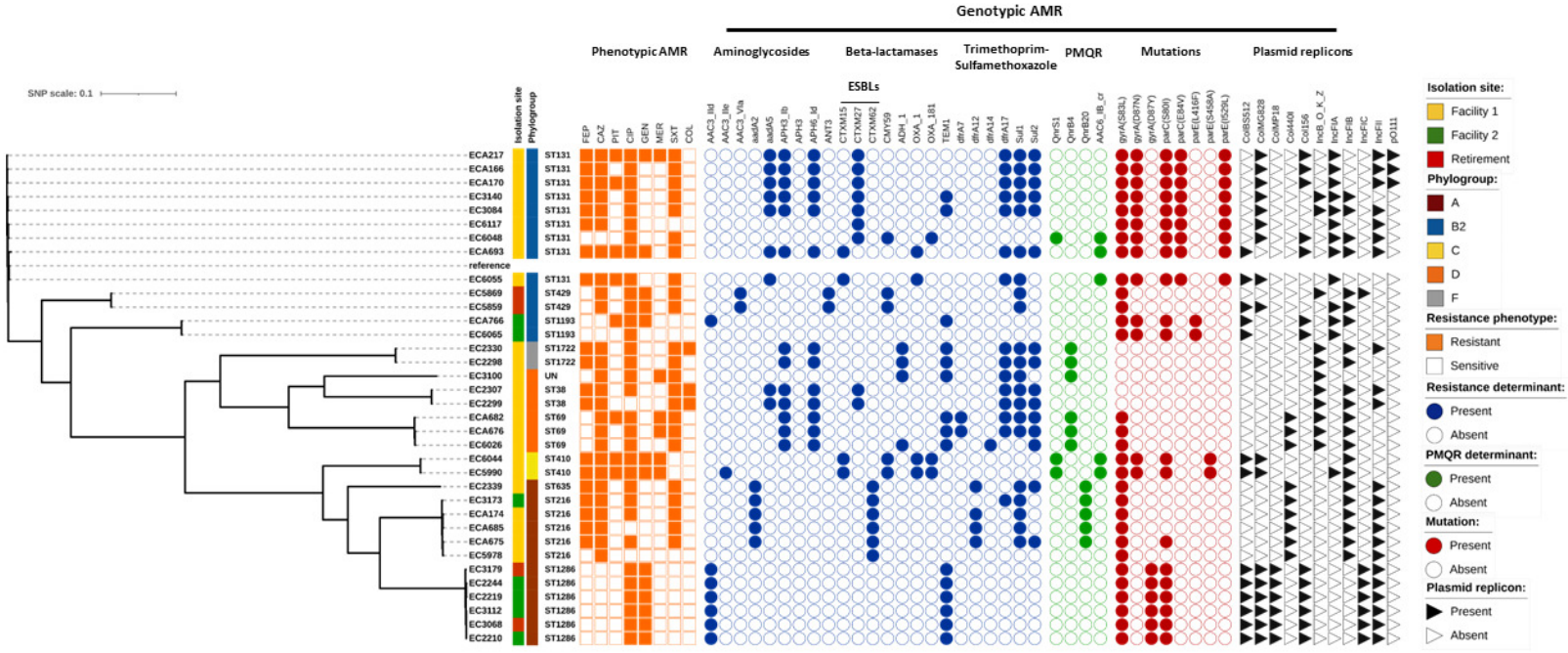
95. ECDC, *Surveillance of antimicrobial resistance in Europe–annual report of the European antimicrobial resistance surveillance network (EARS-Net) 2018*. 2019, European Centre for Disease Prevention and Control Solna, Sweden.
96. Stapleton, A.E., et al., *Escherichia coli Resistance to Fluoroquinolones in Community-Acquired Uncomplicated Urinary Tract Infection in Women: a Systematic Review.* Antimicrobial Agents and Chemotherapy, 2020. **64**(10): p. e00862-20.
97. Wiener, J., et al., *Multiple antibiotic–resistant Klebsiella and Escherichia coli in nursing homes.* Jama, 1999. **281**(6): p. 517-523.
98. Mendelson, G., et al., *Prevalence and risk factors of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in an Israeli long-term care facility.* European journal of clinical microbiology and infectious diseases, 2005. **24**(1): p. 17-22.
99. Maslow, J.N., B. Lee, and E. Lautenbach, *Fluoroquinolone-resistant Escherichia coli carriage in long-term care facility.* Emerging infectious diseases, 2005. **11**(6): p. 889.
100. Jones-Dias, D., et al., *QnrS1- and Aac(6')-Ib-cr-Producing Escherichia coli among Isolates from Animals of Different Sources: Susceptibility and Genomic Characterization.* Frontiers in Microbiology, 2016. **7**.
101. Kücken, D., H.-H. Feucht, and P.-M. Kaulfers, *Association of qacE and qacEΔ1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria.* FEMS Microbiology Letters, 2000. **183**(1): p. 95-98.
102. Amos, G.C.A., et al., *Validated predictive modelling of the environmental resistome.* The ISME Journal, 2015. **9**(6): p. 1467-1476.
103. Dyar, O., L. Pagani, and C. Pulcini, *Strategies and challenges of antimicrobial stewardship in long-term care facilities.* Clinical Microbiology and Infection, 2015. **21**(1): p. 10-19.

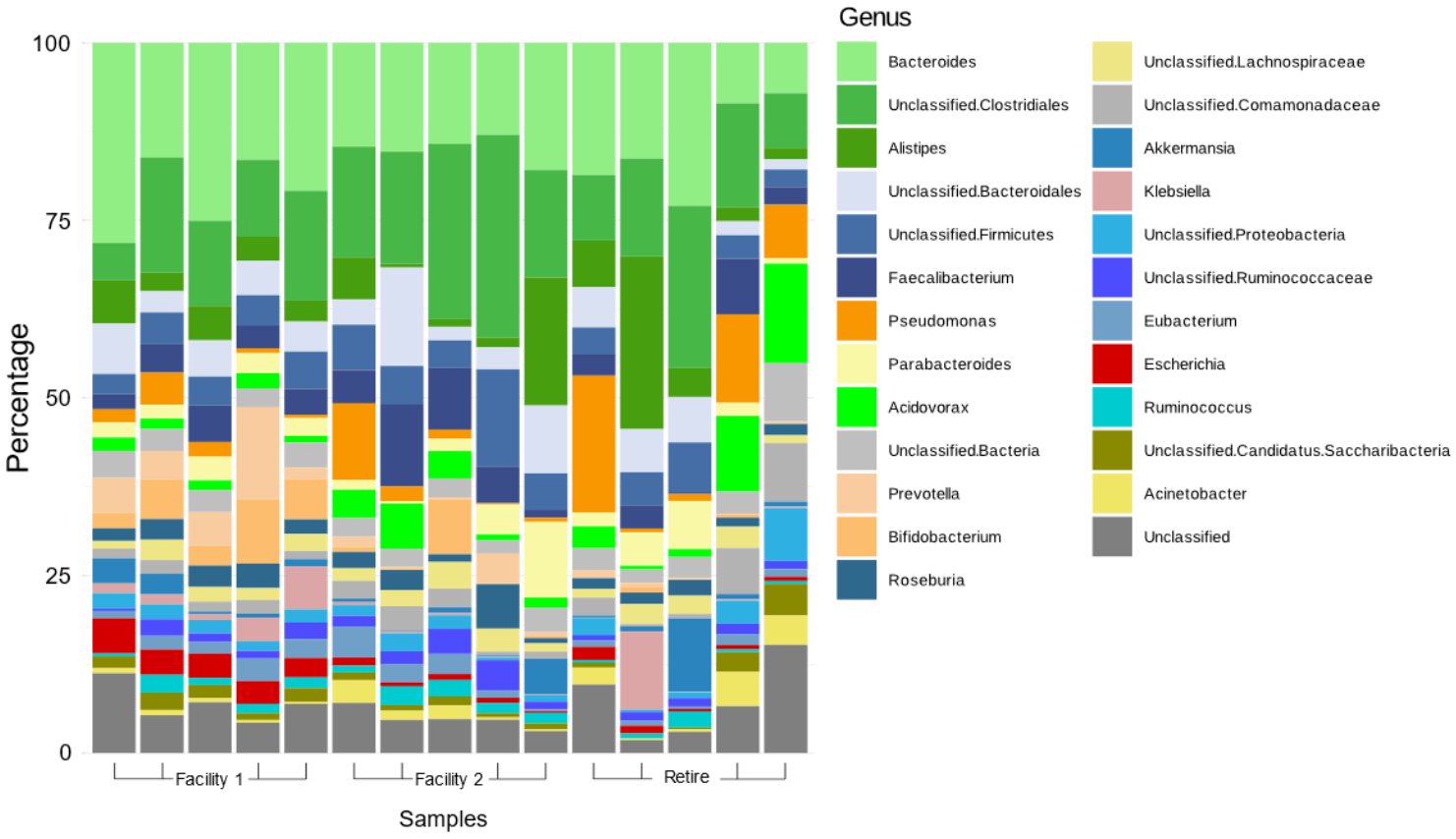

A
Facility 1
Facility 2
Retirement
% resistant E. coli
100
80
60
40
20
0
84.5
7.0
0
98.0
7.0
38.0
23.0
11.0
0
98.0
93.0
100.0
29.0
81.0
100.0
21.0
0
0
83.0
11.0
38.0
7.0
0
0
FEP
CAZ
PIP-TAZ
CIP
GEN
MER
SXT
COL
Antibiotics
B
Number of Isolates
35
30
25
20
15
10
5
0
1 Ab
2 Abs
3 Abs
4 Abs
5 Abs
6 Abs
7 Abs
Facility 1
Facility 2
Retirement
C
Number of Isolates
60
40
20
0
Non-MDR
MDR
Facility 1
Facility 2
Retirement

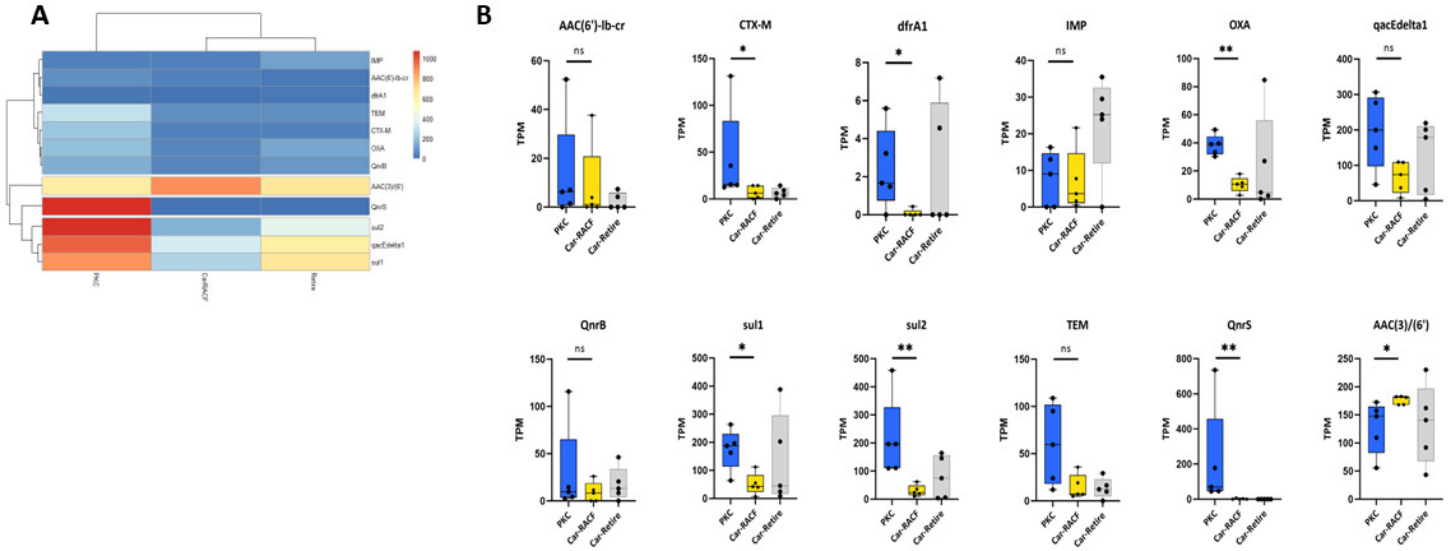

PCA - Biplot
GEN
CIP
MER
PIP.TAZ
FEP
COL
CAZ
SXT
4
2
0
-2
-2.5
0.0
2.5
5.0
Dim2 (27.5%)
Dim1 (30.8%)
Locations
Facility_1
Facility_2
Retirement


Facility 1
8
4
8
17
38
13
8
4
Facility 2
57
29
14
Retirement
50
50
% sequence type
Sequence Types:
1722
1286
1193
635
429
410
216
131
69
38
Unknown


Facility 1
Facility 2
Retirement
10
5
0
Number of E. coli
A
B2
C
D
F
Phylogroup


Genotypic AMR
Phenotypic AMR
Aminoglycosides
Beta-lactamases
ESBLs
Trimethoprim-Sulfamethoxazole
PMQR
Mutations
Plasmid replicons
SNP scale: 0.1
Isolation site
Phylogroup
FEP
CAZ
PIT
CIP
GEN
MER
SXT
COL
AAC3_IId
AAC3_IIe
AAC3_VIa
aadA2
aadA5
APH3_Ib
APH3
APH6_Id
ANT3
CTXM15
CTXM27
CTXM62
CMY59
ADH_1
OXA_1
OXA_181
TEM1
dfrA7
dfrA12
dfrA14
dfrA17
Sul1
Sul2
QnrS1
QnrB4
QnrB20
AAC6_IB_cr
gyrA(S83L)
gyrA(D87N)
gyrA(D87Y)
parC(S80I)
parC(E84V)
parE(L416F)
parE(S458A)
parE(I529L)
ColBS512
ColMG828
ColMP18
Col440I
Col156
IncB_O_K_Z
IncFIA
IncFIB
IncFIC
IncFII
pO111
ECA217 ST131
ECA166 ST131
ECA170 ST131
EC3140 ST131
EC3084 ST131
EC6117 ST131
EC6048 ST131
ECA693 ST131
reference
EC6055 ST131
EC5869 ST429
EC5859 ST429
ECA766 ST1193
EC6065 ST1193
EC2330 ST1722
EC2298 ST1722
EC3100 UN
EC2307 ST38
EC2299 ST38
ECA682 ST69
ECA676 ST69
EC6026 ST69
EC6044 ST410
EC5990 ST410
EC2339 ST635
EC3173 ST216
ECA174 ST216
ECA685 ST216
ECA675 ST216
EC5978 ST216
EC3179 ST1286
EC2244 ST1286
EC2219 ST1286
EC3112 ST1286
EC3068 ST1286
EC2210 ST1286
Isolation site:
Facility 1
Facility 2
Retirement
Phylogroup:
A
B2
C
D
F
Resistance phenotype:
Resistant
Sensitive
Resistance determinant:
Present
Absent
PMQR determinant:
Present
Absent
Mutation:
Present
Absent
Plasmid replicon:
Present
Absent


Genus
Bacteroides
Unclassified.Clostridiales
Alistipes
Unclassified.Bacteroidales
Unclassified.Firmicutes
Faecalibacterium
Pseudomonas
Parabacteroides
Acidovorax
Unclassified.Bacteria
Prevotella
Bifidobacterium
Roseburia
Unclassified.Lachnospiraceae
Unclassified.Comamonadaceae
Akkermansia
Klebsiella
Unclassified.Proteobacteria
Unclassified.Ruminococcaceae
Eubacterium
Escherichia
Ruminococcus
Unclassified.Candidatus.Saccharibacteria
Acinetobacter
Unclassified
Percentage
100
75
50
25
0
Facility 1
Facility 2
Retire
Samples


A
B
IMP
AAC(6')-Ib-cr
dfrA1
TEM
CTX-M
OXA
QnrB
AAC(3)/(6')
QnrS
sul2
qacEdelta1
sul1
PKC
Car-RACF
Retire
1000
800
600
400
200
0
AAC(6')-Ib-cr
CTX-M
dfrA1
IMP
OXA
qacEdelta1
QnrB
sul1
sul2
TEM
QnrS
AAC(3)/(6')
TPM
ns
*
**
PKC
Car-RACF
Car-Retire