Molecular epidemiology of third-generation cephalosporin-resistant Enterobacteriaceae from Southeast Queensland, Australia

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Running title: Population genomics of 3GC-R Enterobacteriaceae

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27 **Synopsis**

28 Third-generation cephalosporin-resistant (3GC-R) Enterobacteriaceae represent a major
29 threat to human health. Here, we captured 288 3GC-R Enterobacteriaceae clinical isolates
30 from 264 patients presenting at a regional Australian hospital over a 14-month period.
31 Alongside routine mass spectrometry speciation and antibiotic sensitivity testing, isolates
32 were examined using rapid (~40 min) real-time PCR assays targeting the most common
33 extended spectrum β-lactamases (ESBLs; CTX-M-1 and CTX-M-9 groups, plus TEM, SHV, and
34 an internal 16S ribosomal DNA control). AmpC CMY β-lactamase prevalence was also
35 examined. *Escherichia coli* (80.2%) and *Klebsiella pneumoniae* (17.0%) were dominant, with
36 *Klebsiella oxytoca*, *Klebsiella aerogenes* and *Enterobacter cloacae* infrequently identified.
37 Ceftriaxone and cefoxitin resistance were identified in 97.0% and 24.5% of *E. coli* and *K.*
38 *pneumoniae* isolates, respectively. Consistent with global findings in Enterobacteriaceae,
39 most (98.3%) isolates harbored at least one β-lactamase gene, with 144 (50%) encoding
40 $bla_{\text{CTX-M-1}}$ group, 92 (31.9%) $bla_{\text{CTX-M-9}}$ group, 48 (16.7%) bla_{SHV}, 133 (46.2%) bla_{TEM}, and 34
41 (11.8%) bla_{CMY}. A subset of isolates (*n*=98) were subjected to whole-genome sequencing
42 (WGS) to identify the presence of cryptic resistance determinants, and to verify genotyping
43 accuracy. WGS of β-lactamase negative or carbapenem-resistant isolates identified
44 uncommon ESBLs and carbapenemases, including bla_{NDM} and bla_{IMP}, and confirmed all PCR-
45 positive genotypes. We demonstrate that our PCR assays enable the rapid and cost-effective
46 identification of ESBLs in the hospital setting, which has important infection control and
47 therapeutic implications.

48 **Introduction**

49 Third-generation cephalosporin-resistant (3GC-R) Enterobacteriaceae represent a significant
50 threat to human health due to their ability to rapidly transmit antimicrobial resistance (AMR)
51 determinants within and among bacterial populations (1). Both infection and colonization
52 with these globally-disseminated organisms are associated with poor clinical outcomes and
53 death (2). 3GC-R Enterobacteriaceae prevalence rates vary considerably among hospitals
54 and countries, with particularly high rates in India, Asia, and the Middle East (3). Australia
55 has historically observed a relatively low frequency of 3GC-R Enterobacteriaceae. However,
56 this pattern may be changing, with an extended spectrum β-lactamase (ESBL) phenotype
57 detected in 13.3% of *E. coli* and 9.8% of *K. pneumoniae* in blood culture isolates in 2018 (4),
58 up from 2013 rates of 7.6% and 6.3%, respectively. Moreover, ceftriaxone resistance was
59 seen in 13.4% of *E. coli* and 9.4% of *K. pneumoniae*, with 86.3% and 82.6% containing CTX-M-
60 type ESBL genes, respectively (4).

61

62 AMR in 3GC-R Enterobacteriaceae is generally conferred by the presence of an ESBL or the
63 over-expression of a chromosomal or plasmid-borne AmpC β-lactamase (5). CTX-M-type
64 ESBLs are currently the most common AMR determinants found in 3GC-R
65 Enterobacteriaceae isolated in Australian hospitals, with $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ groups
66 collectively comprising 86.2% of identified CTX-M-type enzymes (4). Detection of ESBL
67 subtypes is not standard clinical practice; instead, their presence is typically inferred from
68 antimicrobial susceptibility patterns and phenotypic confirmatory tests, which are laborious,
69 costly (~$AUD25/isolate), and time-consuming (>24h) to perform (6). Use of molecular-
70 based diagnostics for AMR determinant detection in the clinical setting would improve
71 patient outcomes by enabling more rapid, directed, and personalized treatment strategies,
72 thereby improving antibiotic stewardship measures and reducing AMR burden. Their utility

73 has already been demonstrated in a number of clinical settings, including for bloodstream
74 infections, pneumonia, and infection control screening (7-9).
75
76 In the current study, we prospectively collected all 3GC-R Enterobacteriaceae identified over
77 a 14-month period at the Sunshine Coast University Hospital, Queensland, Australia, a 450-
78 bed, regional teaching and tertiary hospital. Using large-scale comparative genomics, we
79 developed a novel quadriplex and two singleplex real-time PCR assays to rapidly detect the
80 most commonly identified β-lactamase (including ESBL) genes in 3GC-R Enterobacteriaceae.
81 In association with WGS, we used our diagnostic real-time assays to determine the
82 molecular epidemiology of the β-lactamase genes in our isolate set, and compared their
83 performance with both genotypic and phenotypic typing methods to validate assay
84 accuracy, sensitivity and specificity. Finally, we examined the population structure of our
85 3GC-R Enterobacteriaceae population to identify potential cases of nosocomial spread or
86 outbreak clusters.

87

88 **Materials and Methods**

89

90 **Ethics.** This study was approved by The Prince Charles Hospital Human Resources Ethics
91 Committee (HREC/18/QPCH/110). A waiver of informed consent was granted due to the low
92 to negligible risk associated with this study.

93

94 **Isolate collection.** Clinical specimens from 264 adult and pediatric patients (including ICU
95 patients [*n*=48]) that were culture-positive for 3GC-R Enterobacteriaceae were collected
96 prospectively from July 2017 through September 2018 at the Sunshine Coast University
97 Hospital microbiology laboratory (*n*=288). Multiple isolates were collected from 23 patients,
98 either due to re-infection over the course of the study (*n*=9), multiple samples taken during
99 initial admission (*n*=11), or collected during the course of their hospital stay (*n*=3). Samples
100 were inoculated onto ChromID™ ESBL agar (bioMérieux) and ChromID™ Carba/OXA agar
101 (bioMérieux) and examined for colored colonies at 18-24 hours. An ESBL screening disc test
102 was performed on isolates growing on ESBL agar. Isolates were speciated using the VITEK® 2
103 MALDI-TOF MS with the GN ID card (bioMérieux, Murrarie, QLD, Australia). Isolate
104 information is detailed in Table S1.

105

106 **Susceptibility testing.** Antibiotic susceptibility testing and minimum inhibitory concentration
107 (MIC) values were determined using VITEK® 2 or Etest (bioMérieux) according to the
108 manufacturer's instructions. EUCAST criteria (v.10.0) were used for susceptibility and
109 resistance interpretation.

110

111 **Culture DNA preparation.** For PCR, crude DNA was extracted from all isolates using 5%
112 chelex-100 resin (Bio-Rad Laboratories, Gladesville, NSW, Australia). For a subset of samples

113 (*n*=50), a pinhead-sized amount of culture collected using a sterile 10 µL pipette tip was
114 placed directly into 4 µL of PCR mastermix to facilitate rapid turnaround time. For WGS, DNA
115 was extracted using the Quick-DNA miniprep kit (Zymo Research, Irvine, CA, USA).
116
117 **PCR development and β-lactamase detection.** A quadriplex real-time PCR assay was
118 developed to detect the two major CTX-M ESBL groups ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$) and bla_{SHV}
119 (both ESBL and non-ESBL); a 16S rDNA probe-based assay (10) was included in this
120 quadriplex to verify DNA integrity. Additionally, two singleplex assays were designed to
121 amplify ESBL and non-ESBL bla_{TEM} and bla_{CMY} β-lactamase genes (Figure 1). To identify
122 conserved regions for PCR assay design, all publicly available genetic sequences for $bla_{CTX\text{-}M\text{-}1}$,
123 $bla_{CTX\text{-}M\text{-}9}$, bla_{CMY}, bla_{SHV}, and bla_{TEM} were downloaded from the NCBI Nucleotide database (as
124 of March 2019) and aligned using Clustal Omega v1.2.2. Aligned sequences were visualized
125 in Geneious v11.0.4. Candidate primers and probes were identified in Primer Express v3.0
126 (Applied Biosystems, Foster City, California, USA) or Primer3 v0.4.0 (11) followed by primer
127 dimer and heterodimer assessment using NetPrimer
128 (http://www.premierbiosoft.com/netprimer/).
129
130 Black Hole Quencher probes were used to enable multiplexing in a single PCR well and
131 robust target identification. For the quadriplex assay, the following primers and probes (5' to
132 3'; Macrogen Inc., Geumcheon-gu, Seoul, Republic of Korea) were designed: CTX-1 (CTX1_F:
133 CGAMACGTTCCGTCTCGAC, CTX1_R: CTTTCATCCAYGTCACCAGCTG, CTX1_P1: FAM-
134 CGTGATACCACTTCACCTCGGG-BHQ1), CTX-9 (CTX9_F: CGTCSCGCTCATCGATACC, CTX9_R:
135 CAGCTGCTTTTGCGTTTCACTC, CTX9_P1: HEX-CGCTTTCCAATGTGCAGTACC-BHQ1), and SHV
136 (SHV_F: CCARCGTCTGAGCGCC, SHV_R: TATCGGCGATAAACCAGCCC, SHV_P1: Cy5-
137 CAGCACGGAGCGGATCA-BHQ2). The previously described 16S assay (10) incorporated a
138 different fluorophore on the probe (Texas Red-CACGAGCTGACGACARCCATGCA-BHQ2) to

139 accommodate the quadriplex assay format. Two singleplex assays targeting TEM and CMY
140 were also designed: TEM (TEM_F: TGTGGYGCGGTATTATCCCGT, TEM_R:
141 TGCATAATTCTCTTACTGTCAWGCCA, TEM_P1: Cy5.5-CACCAGTCACAGAAAAGCATCT-BHQ2),
142 CMY (CMY_F: TGGCGTATTGGYGATATGTA, CMY_R: TTATGCACCCATGAGGCTTT, CMY_Pr:
143 FAM-TGGGAGATGCTGAACTGGCC-BHQ1). Coincidentally, the reverse primer sequence for
144 our CMY assay was identical to one designed previously (12).
145
146 The quadriplex PCR was carried out using 0.1 µM 16S probe and primers, 0.2 µM CTX-1 and
147 CTX-9 probes and primers, 0.3 µM SHV primers, and 0.4 µM SHV probe. For the TEM
148 singleplex, 0.2 µM TEM primers and probe were used, and for the CMY singleplex, 0.2 uM
149 primers and 0.4 probe were used. All reactions contained 1X Sso Advanced Universal Probes
150 Supermix (Bio-Rad), primers, probe/s, 1 µL DNA template, and molecular grade H_2O, to a 5
151 µL total volume. PCRs were performed on a CFX96 Touch Real-Time PCR Detection System
152 using white Hard Shell 96-well PCR plates and Microseal 'C' Adhesive seals (Bio-Rad) or 0.2
153 mL white 8-tube PCR strips and optical caps (Bio-Rad). For bacterial cultures, thermocycling
154 conditions comprised an initial 2 min denaturation at 95 °C, followed by 45 cycles of 95 °C
155 for 1 sec and 60 °C for 3 sec (total run time=39 min).
156
157 **LoD and LoQ determination.** Three previously characterized strains (13) that represent the
158 PCR assays developed in this study – MER85 (*K. pneumoniae*), MER110 (*E. coli*), and MER132
159 (*E. coli*) – were subject to total DNA extraction using the Gram-positive bacteria extraction
160 protocol of the Qiagen DNeasy kit (Qiagen, Chadstone Centre, VIC, Australia). DNA quantities
161 were measured on the Qubit 4 fluorimeter (Thermo Fisher Scientific, Seventeen Mile Rocks,
162 Qld, Australia) using the Qubit dsDNA BR kit, and normalized to 40 ng/µL in molecular grade
163 H_2O. LoD and LoQ values were determined across 1:10 serial dilutions (range: 40 ng to 0.4
164 fg) using MER85 (bla_{TEM} singleplex), MER110 (bla_{CMY} singleplex), and a 50:50 mix of MER85

165 ($bla_{CTX-M-1}$ group, bla_{SHV}) and MER132 ($bla_{CTX-M-9}$ group) for the quadriplex assay. For PCR
166 thermocycling, denaturation and annealing times were increased to 5 sec and 10 sec,
167 respectively, to ensure assay robustness under very low DNA input conditions. Genome
168 equivalents were calculated by assuming *E. coli* and *K. pneumoniae* genome sizes of 4.984
169 Mbp and 5.638 Mbp, respectively.
170
171 **Assay specificity.** The quadriplex and singleplex assay amplicons were first assessed for
172 specificity *in silico* using the microbial nucleotide discontiguous BLAST tool and the Complete
173 genomes database (http://blast.ncbi.nlm.nih.gov/; performed March 2019). Next, assay
174 specificity was examined by laboratory testing against a range of both pathogenic and non-
175 pathogenic microbial species found humans. This diversity panel comprised *Achromobacter*
176 spp. (*n*=4), *Bordetella bronchiseptica* (*n*=1), *Candida albicans* (*n*=1), *Cupriavidus* sp. (*n*=2),
177 *Enterobacter aerogenes* (*n*=1), *Enterobacter cloacae* (*n*=3), *Enterococcus faecalis* (*n*=1),
178 *Klebsiella pneumoniae* (*n*=2), *Klebsiella oxytoca* (*n*=1), *Lactobacillus* spp. (*n*=2), *Prevotella*
179 spp. (*n*=6), *Pseudomonas aeruginosa* (*n*=10), *Staphylococcus aureus* (*n*=4), *Staphylococcus*
180 *epidermidis* (*n*=1), *Stenotrophomonas maltophilia* (*n*=5), and *Veillonella* spp. (*n*=4).
181
182 **Whole-genome sequencing (WGS).** WGS was performed at the Australian Centre of
183 Ecogenomics (University of Queensland, St Lucia, QLD, Australia) on the NextSeq 500
184 (Illumina, San Diego, CA, USA) with Nextera DNA Flex libraries (Illumina).
185
186 **Genomic analysis.** All genomic data were lightly quality-filtered using Trimmomatic v0.39
187 (14) using parameters described elsewhere (15). WGS data were screened for multiple
188 species using Kraken 2 v2.0.8 (16). Single species were assembled using MGAP v1.1
189 (https://github.com/dsarov/MGAP---Microbial-Genome-Assembler-Pipeline), whereas WGS
190 data containing multiple species were assembled with metaSPAdes (17). To determine

191 plasmid copy number and identity, plasmids were assembled using the plasmid module of
192 SPAdes(18) with plasmid homology determined using BLAST(19). Assemblies were binned
193 with MaxBin v2.0 (20) and speciated with Kraken 2 v2.0.8 (16). Antibiotic resistance genes
194 were identified using the Resistance Gene Identifier (21). Phylogenomic analysis was
195 performed using SPANDx v3.2.1 (22). Samples identified as containing multiple strains
196 according to the method described by Aziz and colleagues (23) were excluded from
197 phylogenetic analysis (*n*=7); samples able to be split into single species using MaxBin were
198 included in phylogenetic analysis (*n*=4). *E. coli* O157:H7 Sakai (NCBI Assembly ID: ASM886v2)
199 (24) or *K. pneumoniae* HS11286 (NCBI Assembly ID: ASM24018v2) (25) were used as read
200 mapping reference genomes for species-specific analyses. To achieve the highest possible
201 resolution, error-corrected assemblies of strains with matching multilocus sequence types
202 (STs) were used as reference genomes for transmission analyses. ST profiles were
203 determined using SRST2 v0.2.0 (26). Phylogenetic trees were reconstructed using PAUP
204 v4.4.7 (27) and visualized in iTOL v5.5(28).
205
206 **WGS data.** Raw sequence reads generated in this study are available on the NCBI Sequence
207 Read Archive database under BioProject accession number PRJNA606985.

208 **Results and Discussion**

209 ESBL enzymes have established themselves as major contributors of AMR in Gram-negative
210 bacteria and are increasing in prevalence, particularly in the community setting. Over 14
211 months, we collected a total of 288 isolates from all diagnosed cases of 3GC-R
212 Enterobacteriaceae in a regional hospital in Southeast Queensland, Australia. Our patient
213 cohort was predominantly female (64.0%) and aged over 65 (60.9%). ICU patient specimens
214 comprised 54 (18.8%) of the total collection, and were obtained from 48 ICU patients over a
215 12-month period. Five ICU patients had multiple isolates collected over the course of their
216 stay in the ICU. Three patients isolated single species with identical AMR profiles, two
217 patients isolated multiple species: one with identical AMR profiles and one with differing
218 profiles (Table S1). *E. coli* and *K. pneumoniae* were dominant, comprising 80.2% and 17.0%
219 3GC-R strains, respectively (Table 1 and S1). The remaining isolates were identified as *E.*
220 *cloacae* (1.4%; *n*=4), *K. oxytoca* (1.0%; *n*=3), and *K. aerogenes* (0.3%; *n*=1). Resistance to
221 ceftriaxone, ceftazidime or cefepime was common and observed in 96.2%, 93.4%, and 86.8%
222 of isolates, respectively. In contrast, cefoxitin resistance was noted in only 81 (28.1%)
223 isolates (Table 1). Meropenem resistance was rare, with only six resistant strains (2.1%)
224 identified. The double disc diffusion method for ESBL phenotypic testing was negative in 32
225 (11.1%) isolates, indicating a lack of β-lactamase inhibition in the presence clavulanic acid.
226 The remaining 256 (88.9%) isolates were positive for this phenotypic test.

227

228 *ESBL and β-lactamase assay performance.* Preliminary validation of our singleplex (bla_{TEM},
229 bla_{CMY}) and quadriplex ($bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{SHV}, 16S rDNA) PCR assays was
230 performed to determine their accuracy and specificity. Following *in silico* verification, assays
231 were tested on 12 previously characterized control strains (Table S2) (29) that represented a
232 range of Enterobacteriaceae containing the gene targets of interest. All expected targets
233 were identified in this control dataset; no false positives or false negatives were detected,

234 although both the *bla*$_{TEM}$ and 16S rDNA assays yielded late (>30 cycles) amplification in no-
235 template control wells (Figure 1). This result was expected due to residual *E. coli* DNA
236 presence in the recombinant Sso Advanced *Taq* polymerase, which encodes both *bla*$_{TEM}$ and
237 16S rRNA genes.
238
239 We found that all genotypes could be accurately determined for the control strains by
240 directly inoculating PCR wells with a very small amount of bacterial culture, thereby
241 obviating the need for DNA extraction and leading to faster time-to-diagnosis. However, this
242 technique requires skill to ensure that the PCR is not inhibited by excess DNA and cellular
243 constituents, and can lead to greater PCR failures if not performed correctly. In addition, we
244 recommend using 10 µL rather than 5 µL total PCR volume as, in our hands, the lower
245 reaction volume led to a greater failure rate. Inclusion of the 16S rDNA assay enables true
246 negatives to be differentiated from PCR failures. This method provides an attractive option
247 where rapid diagnosis is paramount, or where DNA extraction is not feasible.
248
249 Next, we screened our PCR assays across a diversity panel of non-Enterobacteriaceae human
250 pathogens or colonizers (*n*=48 strains). Our assays showed 100% sensitivity and specificity
251 for all targets, with no amplification detected in these isolates, except for the 16S target in
252 bacterial strains; as with the control dataset, the *bla*$_{TEM}$ PCR was inhibited by the presence of
253 non-*bla*$_{TEM}$-encoding DNA and therefore did not amplify in any diversity panel strains.
254
255 *LoQ and LoD determination of the ESBL and β-lactamase assays.* Using serial dilutions of
256 previously characterized DNA samples ranging from 40 ng to 0.4 fg, the LoQ values for each
257 assay were identified (Figure 2) as follows: *bla*$_{CMY}$, 400 fg (~73 genome equivalents [GEs]);
258 *bla*$_{TEM}$, 400 fg (~65 GEs); 16S rDNA, 40 fg (~3 GEs); *bla*$_{CTX-M-1}$ group, 4 pg (~344 GEs); *bla*$_{CTX-M-9}$
259 group, 4 pg (~344 GEs); and *bla*$_{SHV}$, 4 pg (~344 GEs). LoD values for each assay were: *bla*$_{CMY}$, 4

260 fg (~7 GEs); *bla*$_{CTX-M-1}$ group, 4 pg (~344 GEs); *bla*$_{CTX-M-9}$ group, 400 fg (~34 GEs); and *bla*$_{SHV}$,
261 400 fg (~34 GEs). Due to low levels of recombinant *E. coli* DNA present in the Sso Advanced
262 *Taq* polymerase, which results in late amplification in no-template control wells, LoD values
263 for the *bla*$_{TEM}$ and 16S rDNA assays could not be determined. As expected, these results
264 indicate inferior performance of the quadriplex assay when compared with the singleplex
265 assays. Although not examined in this study, increased LoQ and LoD values may be achieved
266 for the *bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-9}$ group, and *bla*$_{SHV}$ assays by omitting the 16 rDNA assay, or
267 by running these assays in singleplex format. Although we have defined the limits of our
268 assays using pure bacterial templates, it remains to be determined what detection limits
269 would be achieved from clinical specimens.
270
271 *ESBL and β-lactamase prevalence in clinical 3GC-R Enterobacteriaceae isolates.* Following
272 specificity confirmation, the PCR assays were tested across our clinical set (*n*=288). All assays
273 performed well, with 100% concordance between PCR genotypes and WGS of 98 isolates,
274 and with no false negatives identified (Table S3). Overall, a β-lactamase genotype was
275 identified in 277 (96.2%) isolates. Consistent with previously reported rates (29), *bla*$_{CTX-M-1}$
276 group was most common, being identified in 144/288 (50%) isolates, including 105/231
277 (45.5%) *E. coli* and 36/49 (73.5%) *K. pneumoniae*. The *bla*$_{CTX-M-9}$ group was next frequent,
278 being found in 93/288 (32.3%) isolates, including 89/231 (38.5%) *E. coli* and 4/49 (8.2%) *K.*
279 *pneumoniae*. The presence of both *bla*$_{CTX-M-1}$ group and *bla*$_{CTX-M-9}$ group enzymes was
280 identified in five (1.7%) *E. coli* and one (2.0%) *K. pneumoniae* isolate. Of the eight isolates
281 belonging to other species, *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$ group genes were present in 4/8 (50%)
282 and 0/8 (0%) of these isolates, respectively. The acquired *ampC* gene, *bla*$_{CMY}$, was identified
283 in 30/231 (13%) of *E. coli*, 3/49 (6.1%) of *K. pneumoniae*, and 1/8 (13%) other
284 Enterobacteriaceae species. *bla*$_{CMY}$ and *bla*$_{CTX-M-1}$ were both identified in seven isolates (five

285 *E. coli*, one *K. pneumoniae*, and one *E. cloacae*) and bla_{CMY} and $bla_{CTX\text{-}M\text{-}9}$ were present in two
286 *E. coli* isolates only (Table 1).
287
288 Seven strains (five *E. coli* and two *K. pneumoniae*) had bla_{SHV} only; nine *E. coli* had bla_{TEM}
289 only, and seven (five *K. pneumoniae*, one *E. coli*, and one *E. cloacae*) had both bla_{SHV} and
290 bla_{TEM}. Although most SHV (e.g. $bla_{SHV\text{-}1}$) and TEM (e.g. $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}2}$) enzymes do not
291 typically cause 3GC-R, their presence in strains that lack CTX-M-type ESBLs should be
292 investigated, as these enzymes can occasionally confer 3GC-R through enzyme active site
293 mutation or gene overexpression (5). Of the 14 strains encoding bla_{SHV} but not $bla_{CTX\text{-}M\text{-}1}$
294 group, $bla_{CTX\text{-}M\text{-}9}$ group, or bla_{CMY} enzymes, five had accompanying WGS data, which
295 permitted assessment of bla_{SHV} ESBL status. In all cases, the bla_{SHV} variant was an ESBL (two
296 $bla_{SHV\text{-}2}$, one $bla_{SHV\text{-}64}$, one $bla_{SHV\text{-}66}$, and one $bla_{SHV\text{-}134}$) (30). In contrast, none of the four
297 strains encoding bla_{TEM} but not $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CMY}, or ESBL-encoding
298 bla_{SHV} enzymes were ESBLs, with all being $bla_{TEM\text{-}1}$. Three of these were negative with the
299 double disc diffusion method, indicating probable chromosomal mutations underpinning this
300 phenotype. The basis for the ESBL phenotype in the fourth strain could not be elucidated.
301
302
303 *Correlation between* bla_{CMY} *and cefoxitin resistance*. Of the 81 cefoxitin-resistant isolates,
304 bla_{CMY} was detected in only 31 (38.3%) cases. Although imperfect, cefoxitin resistance is
305 often used as a marker for AmpC presence (32). Correlation is also dependent on the MIC,
306 with higher MICs more reliably predicting AmpC presence (32). Our findings concur with this
307 prior work, with 61.7% of cefoxitin-resistant isolates failing to amplify bla_{CMY}. AmpC has
308 previously been reported as an underappreciated mechanism of 3GC-R due to a lack of
309 standardized testing (33). As such, molecular methods that can detect other common and
310 emerging β-lactamase gene families (e.g. bla_{DHA}, bla_{OXY}, bla_{MIR}) may prove useful for infection

311 control management and treatment of multidrug-resistant infections. This is particularly true
312 for piperacillin-tazobactam, which may not be effective against AmpC producers (29, 34).
313
314 *WGS identifies uncommon ESBL genes.* Eleven (3.8%) PCR-negative isolates that otherwise
315 successfully amplified their 16S rDNA internal control (seven *E. coli*, one *K. pneumoniae*, one
316 *K. oxytoca*, one *K. aerogenes* and one *E. cloacae*) were subjected to WGS to determine the
317 molecular basis of 3GC-R. Six were found to carry uncommon β-lactamase enzymes, with
318 three *E. coli* encoding bla_{DHA-1}, one *K. pneumoniae/K. oxytoca* mixture harboring $bla_{OXY-2-4}$,
319 one *K. oxytoca* carrying $bla_{OXY-2-1}$, and one *E. cloacae* carrying bla_{ACT-17}. The remaining five
320 isolates had no discernable 3GC-R determinants, although misclassification via automated
321 susceptibility testing or upregulation of chromosomal β-lactamases are both possible causes
322 for this discrepancy.
323
324 Six isolates (three *E. cloacae*, two *E. coli* and one *K. pneumoniae*) exhibiting meropenem
325 resistance alongside 3GC-R were also subjected to WGS to determine the basis of resistance
326 towards this carbapenem. As expected, meropenem resistance was mostly conferred by
327 carbapenemases, with 4/6 (66.6%) isolates possessing one of these enzymes. bla_{NDM-1} or
328 bla_{IMP-4} were identified in two *E. cloacae* isolates, bla_{NDM-5} was identified in an *E. coli* strain,
329 and an OXA-48-like enzyme, $bla_{OXA-232}$, was identified in a *K. pneumoniae* isolate. No
330 carbapenemase gene could be identified in the remaining two isolates (one *E. coli* and one *E.*
331 *cloacae*). Although not investigated in this study, chromosomally-encoded porin loss or
332 efflux pump overexpression may form the basis of meropenem resistance in these strains.
333
334 *Monitoring patient-to-patient spread in an ICU.* Fifty-four (18.8%) 3GC-R isolates obtained
335 during this study originated from ICU-admitted patients. Due to their dominance, only *E. coli*
336 and *K. pneumoniae* genomes were analyzed for potential transmission events. Among the *E.*

337 *coli* population (Figure 3), isolates SCHI0016.S.60 and SCHI0016.S.62, and SCHI0016.S.265
338 and SCHI0016.S.46, were separated by 10 SNPs and 2 SNPs, respectively; however, both
339 pairs came from individual patients, reflecting expected levels of within-host evolution of a
340 single infecting strain. *E. coli* isolates SCHI0016.S.42 and SCHI0016.S.294 were very similar,
341 being separated by 13 SNPs. Although both strains were isolated from ICU-admitted
342 patients, SCHI0016.S.294 was isolated 306 days after SCHI0016.S.42 with no other linking
343 epidemiology identified. Additionally, no similar strains were isolated from ICU patients over
344 this time period suggesting that these do not represent transmission. *E. coli* isolates
345 SCHI0016.S.172 and SCHI0016.S.274 differed by ~300 SNPs, with no epidemiological or
346 temporal correlation. ~300 SNPs separated SCHI0016.S.112 and SCHI0016.S.229, again with
347 no demonstrable epidemiological link. Among *K. pneumoniae* (Figure 4), the closest isolates,
348 SCHI0016.S.15 and SCHI0016.S.261, differed by 361 SNPs, with no epidemiological link.
349 Additionally, no horizontal transfer of plasmids carrying ESBL determinants was observed. As
350 standard practice, all ICU patients, and all patients in other wards infected or colonized with
351 ESBL-producing *E. coli* and *K. pneumoniae*, have single rooms with an assigned nurse (1:1
352 ratio), with contact precautions exercised. In addition, hand hygiene rates in our hospital ICU
353 are generally above 80% (35). Taken together, our results demonstrate no evidence of
354 transmission in the ICU, suggesting that the implemented hygiene measures are effective.
355
356 *Population structure of E. coli and K. pneumoniae genomes.* We subjected an additional 36
357 strains to WGS (total *n*=98) to capture a snapshot of genomic diversity and population
358 structure (Figures 3 and 4). *In silico* MLST of the 79 *E. coli* strains found that ST131 was most
359 common (*n*=11; 13.9%). This finding is consistent with a recent study of the population
360 structure of Australian, New Zealand, and Singaporean multidrug-resistant *E. coli* isolates
361 (29). The highly successful ST131 clone has now spread worldwide, where it frequently
362 causes urinary tract and bloodstream infections and accounts for 12–27% of all community-

363 acquired *E. coli* infections (36, 37). Among our ST131 isolates, we found high frequencies of
364 fluoroquinolone resistance-conferring variants ($GyrA_{S83L}$, $GyrA_{D87N}$, $ParC_{S80I}$ and $ParC_{E84V}$)
365 alongside frequent carriage of plasmid-borne CTX-M-14 (CTX-M-9 group) and CTX-M-15
366 (CTX-M-1 group) ESBLs. We also identified three strains belonging to the emerging multi-
367 drug resistant pandemic clone, ST1193. These isolates also possessed the same three
368 fluoroquinolone-resistant SNPs as ST131. It has been proposed that ST1193 strains evolved
369 fluoroquinolone resistance relatively recently through multiple recombination events with
370 other *E. coli* strains (38).
371
372 Phylogenomic analysis of our 10 *K. pneumoniae* genomes identified several distinct lineages,
373 which comprised two ST14 strains and one each of ST17, ST307, ST309, ST395, ST656, ST697,
374 ST873, and ST969. Of note, the globally disseminated ST258 clone, which is responsible for a
375 large number of hospital deaths (39), was not identified in our dataset. However, we
376 identified another globally disseminated clone, ST395, which has also been retrieved from
377 clinical cases in Europe and Asia (40). Concerningly, this ST395 isolate, SCHI0016.S.137,
378 carried both the ESBL genes bla_{SHV-11} and $bla_{CTX-M-15}$ and the OXA-48-like carbapenemase,
379 $bla_{OXA-232}$, the latter of which confers low-level meropenem resistance. Additionally, we
380 observed one *K. pneumoniae* ST307, which is an emerging multi-drug resistant, globally
381 disseminated clone postulated to have emerged in the mid-1990s(41). Although reports of
382 this clone from Australian populations in uncommon it has been previously observed(41).
383
384 This study had several recognized limitations. First, isolates were collected from a
385 nosocomial setting; no community sources were sampled. It is well-established that
386 differences in AMR patterns and their associated molecular mechanisms exist between
387 hospital and community settings. Second, the absence of certain high-risk populations within
388 our hospital (e.g. solid organ or hematopoietic stem cell transplantation) limits the

389 generalizability of our findings to other settings. Third, our PCR assays can only identify the
390 presence or change in copy number of the most common ESBL and *ampC* genes. Although
391 these loci make up the majority (>90%) of β-lactamase enzymes present in 3GC-R
392 Enterobacteriaceae, they do not capture all potential ESBLs, leaving less common ESBL and
393 *ampC* genes unidentified. In addition, PCR assays for bla_{TEM} or bla_{SHV} cannot discriminate
394 between ESBLs and non-ESBLs, with additional assays or concurrent WGS required to
395 confirm ESBL status. Our assays also cannot detect chromosomal changes leading to AMR
396 (e.g. porin mutations or efflux pump up-regulation), making these assays less useful for 3GC-
397 R bacteria that frequently encode these AMR determinants (e.g. *Pseudomonas aeruginosa*).
398 Fourth, our PCR was unable to distinguish chromosomal vs. low-copy plasmid-borne β-
399 lactamase genes, which has implications for AMR transmissibility between and among
400 bacterial populations. Finally, ~40% of samples were obtained from rectal swabs, which have
401 limitations for detection of multidrug-resistant organisms due to the high microbial burden
402 coupled with difficulties in sample collection. It is therefore likely that we did not capture all
403 3GC-R isolates during our study period, and thus may have missed potential transmission
404 events.
405

Conclusion

Taken together, our findings identified a predominance of CTX-M (and to a lesser extent, CMY) β-lactamases among 3GC-R Enterobacteriaceae in the Sunshine Coast region. These findings are consistent with global findings of Gram-negative bacterial AMR determinants, particularly among *E. coli*. Our study demonstrates the likely absence of 3GC-R Enterobacteriaceae transmission in our ICU setting, indicating effective infection control processes. We show that rapid molecular assays, such as our multiplex PCR, can detect common β-lactamase genes from bacterial culture within 40 minutes. These early molecular results would permit more informed selection of antibiotic therapy up to 1-2 days prior to 3GC-R confirmation with traditional culture methodologies. Further research is required to determine whether these molecular assays can be used directly on clinical specimens, and whether the implementation of molecular assays and more advanced technologies (e.g. WGS) is associated with improved patient outcomes in the hospital setting.

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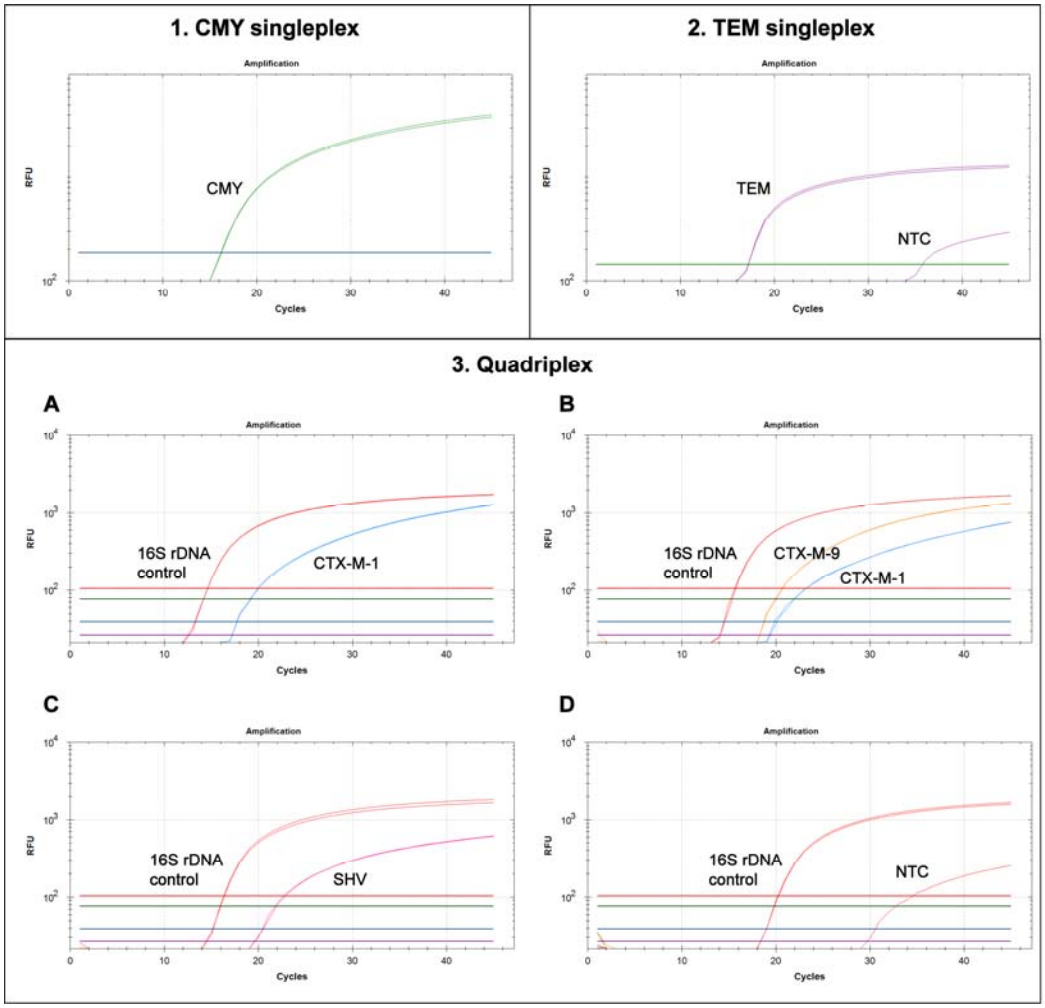
430 **Figures**



431

432 **Figure 1: Singleplex and quadriplex PCR performance on third-generation cephalosporin-**

433 **resistant Enterobacteriaceae.** 1: *Enterobacter cloacae* SCHI0016.S.104 containing CMY. 2:

434 *Escherichia coli* SCHI0016.S.205 containing TEM. 3A: *E. coli* SCHI0016.S.187 positive for CTX-

435 M group 1. 3B: *E. coli* SCHI0016.S.60 positive for both CTX-M groups 1 and 9. 3C: *E. coli*

436 SCHI0046.S.46. 3D: *E. coli* with only 16s rDNA amplification with no CTX-M or SHV detected.

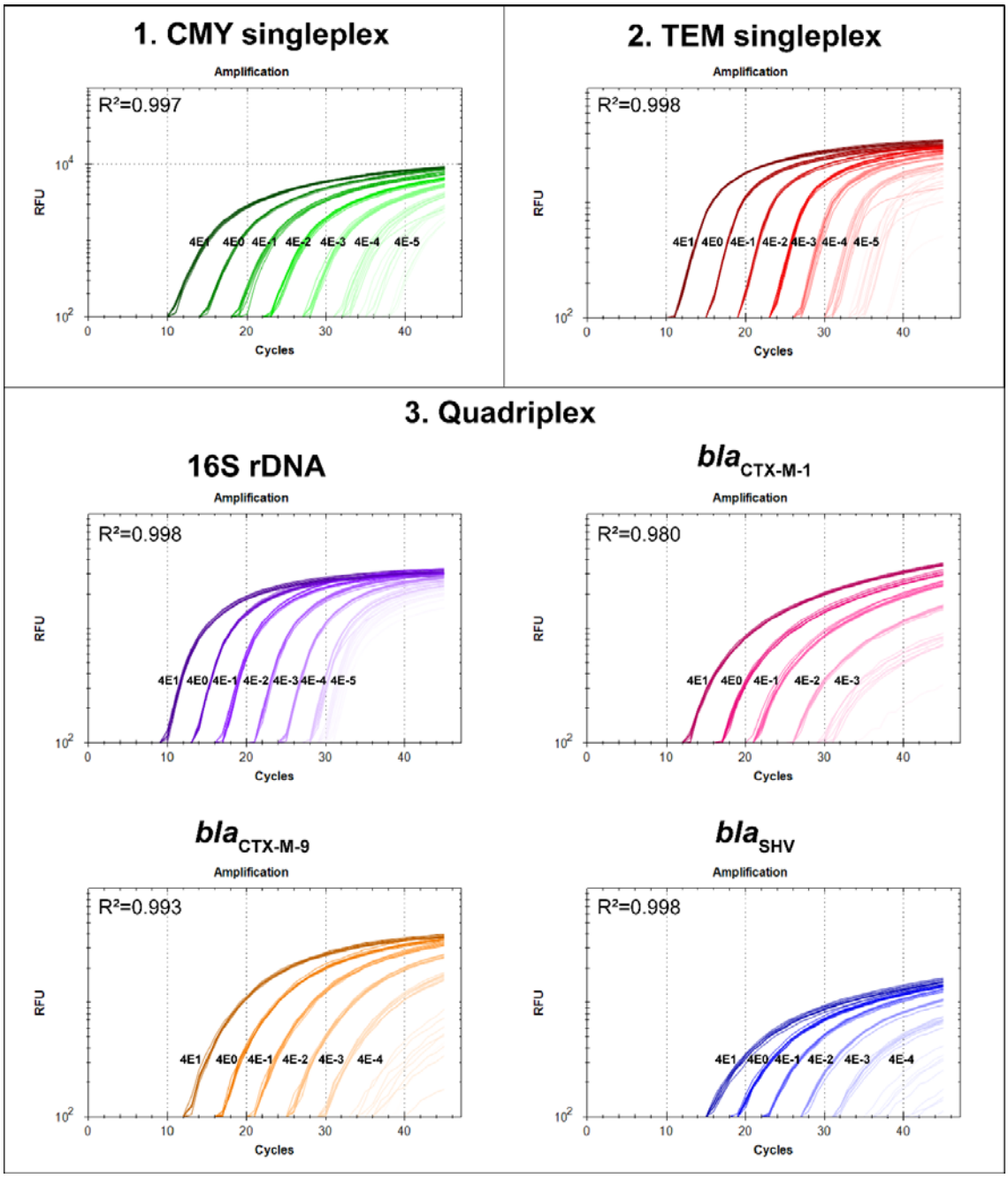
437 Note that both 16S rDNA and the TEM assays have late amplification in the no-template

438 controls due to low levels of recombinant *E. coli* DNA present in the Sso Advanced *Taq*

439 polymerase.

440

441



442

443 **Figure 2. Limits of Detection (LoD) and Quantitation (LoQ) for the extended spectrum β-**

444 **lactamase real-time PCR assays developed in this study.** The bla_{CMY} and bla_{TEM} assays were

445 examined as singleplex PCRs, whereas the 16S rDNA, $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, and

446 bla_{SHV} assays were examined in quadriplex format.

447

Figure 3. Whole-genome phylogeny of 79 third-generation cephalosporin-resistant *Escherichia coli* isolates and their β-lactamase gene complement. Sequence type (ST)131 is indicated by the gray-shaded box; strains isolated from intensive care unit patients are

452 highlighted in green.

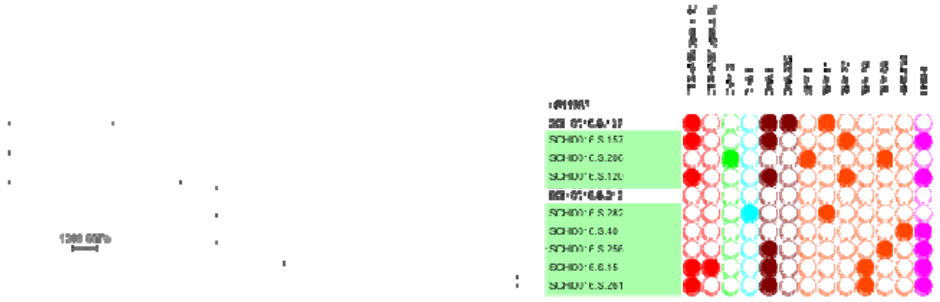


Figure 4. Whole-genome phylogeny and β-lactamase enzyme presence in *Klebsiella pneumoniae*. Green-shaded isolates were isolated from the intensive care unit.

Transparency declarations

Dr. Harris reports research grants from Shionogi, Sandoz, and MSD and speaker's fees from Pfizer, outside the submitted work. No conflicts are declared for remaining authors

462
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617

618

619

621 **Tables**

622

623 **Table 1:** Summary of antimicrobial resistance mechanisms among 288 clinical Enterobacteriaceae isolates retrieved from 264 patients admitted to a
624 Southeast Queensland hospital over a 14-month period.

Organism	***E. coli* (*n*=231)**	***K. pneumoniae* (*n*=49)**	***K. aerogenes (n=1)***	***K. oxytoca* (*n*=3)**	***E. cloacae* (*n*=4)**
Phenotypic antimicrobial resistance[a]					
Ceftriaxone	223 (96.5%)	46 (93.9%)	1 (100%)	3 (100%)	4 (100%)
Ceftazidime	215 (93.1%)	47 (95.9%)	1 (100%)	2 (66.7%)	4 (100%)
Cefepime	201 (87.0%)	43 (87.8%)	1 (100%)	2 (66.7%)	3 (75.0%)
Cefoxitin	64 (27.7%)	12 (24.5%)	1 (100%)	0 (0%)	4 (100%)
Piperacillin-tazobactam	65 (28.1%)	31 (63.3%)	1 (100%)	3 (100%)	4 (100%)
Ticarcillin-clavulanate	186 (80.5%)	46 (93. 9%)	0 (0%)	3 (100%)	3 (75.0%)
Meropenem	3 (1.3%)	2 (4.1%)	0 (0%)	0 (0%)	3 (75.0%)
ESBL phenotypic test[b]					
Positive	204 (88.3%)	46 (93.9%)	0 (0%)	2 (66.7%)	4 (100%)
Negative	27 (11.7%)	3 (6.1%)	1 (100%)	1 (33.3%)	0 (0%)
ESBL and *ampC* gene prevalence					
$bla_{\text{CTX-M-1}}$	104 (45.0%)	36 (73.5%)	0 (0%)	2 (66.7%)	2 (50.0%)
$bla_{\text{CTX-M-9}}$	89 (38.5%)	4 (8.2%)	0 (0%)	0 (0%)	0 (0%)
bla_{SHV}	9 (3.9%)	38 (77.6%)	0 (0%)	0 (0%)	1 (25.0%)
bla_{TEM}	93 (40.3%)	33 (67.3%)	0 (0%)	2 (66.7%)	2 (50.0%)
bla_{CMY}	30 (13.0%)	3 (6.1%)	0 (0%)	0 (0%)	1 (25.0%)
PCR-negative[c]	7 (3.0%)	1 (4%)	1 (100%)	1 (33.3%)	1 (25.0%)

625 [a]Antimicrobial susceptibility testing performed by automated method (VITEK 2®) and EUCAST breakpoints applied
626 [b]ESBL phenotypic testing performed by disc diffusion method
627 [c]DNA integrity was confirmed by 16S rDNA PCR (10), which forms part of the quadriplex assay. Three PCR negative *E. coli* strains encoded $bla_{\text{DHA-1}}$, one *E.*
628 *cloacae* encoded $bla_{\text{ACT-17}}$, one *K. oxytoca* encoded a $bla_{\text{OXY-2-1}}$, a second *K. oxytoca* (included as a mixture with *K. pneumoniae*) encoded $bla_{\text{OXY-2-4}}$, and 5/11
629 isolates had no β-lactamases identified despite whole-genome sequencing.