Using whole-genome sequencing and a pentaplex real-time PCR to characterize third-generation cephalosporin-resistant Enterobacteriaceae from Southeast Queensland, Australia

Stewart AG[1,2], Price EP[3,4], Schabacker K[3,4], Birikmen M[3,4], Harris PNA[2,5], Choong K[1], Subedi S[1,6] and Sarovich DS[3,4#]

[1]Department of Infectious Diseases, Sunshine Coast University Hospital, Birtinya, QLD, Australia

[2]Centre for Clinical Research, University of Queensland, Herston, QLD, Australia

[3]GeneCology Research Centre, University of the Sunshine Coast, Sippy Downs, QLD, Australia

[4]Sunshine Coast Health Institute, Birtinya, QLD, Australia

[5]Pathology Queensland, Central Microbiology, Royal Brisbane and Women's Hospital, Herston, QLD, Australia

[6]Department of Microbiology, Pathology Queensland, Sunshine Coast University Hospital, Birtinya, QLD Australia

Keywords: Antibiotic, antimicrobial, resistance, cephalosporin, ESBL, whole-genome sequencing, real-time PCR, Enterobacteriaceae

Running title: Population genomics of 3GC-R Enterobacteriaceae

#Corresponding author

Derek S. Sarovich

dsarovich@usc.edu.au

+61754563748

University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, QLD, 4558, Australia

Abstract

Third-generation cephalosporin-resistant (3GC-R) Enterobacteriaceae represent a major threat to human health. Here, we captured 288 3GC-R Enterobacteriaceae clinical isolates from 258 patients presenting at a regional Australian hospital over a 14-month period. Alongside routine mass spectrometry speciation and antibiotic sensitivity testing, isolates were examined using a rapid (~40 min) pentaplex real-time PCR assay targeting the most common extended spectrum β-lactamases (ESBLs; CTX-M-1 and CTX-M-9 groups, plus TEM, SHV, and an internal 16S ribosomal DNA control). Additionally, AmpC CMY β-lactamase prevalence was examined using a singleplex PCR. A subset of isolates, including all 3GC-R isolates obtained from the intensive care unit, were subjected to whole-genome sequencing (WGS) to assess transmission dynamics, the presence of unidentified resistance determinants, and genotyping accuracy. *Escherichia coli* (80.2%) and *Klebsiella pneumoniae* (17.0%) were dominant, with *Klebsiella oxytoca*, *Klebsiella aerogenes* and *Enterobacter cloacae* infrequently identified. Ceftriaxone and cefoxitin resistance was identified in 97% and 24.5% of *E. coli* and *K. pneumoniae* isolates, respectively. Consistent with global findings in Enterobacteriaceae, the majority (98.3%) of isolates harbored at least one β-lactamase gene, with 144 (50%) encoding $bla_{\text{CTX-M-1}}$ group, 92 (31.9%) $bla_{\text{CTX-M-9}}$ group, 48 (16.7%) bla_{SHV}, 133 (46.2%) bla_{TEM}, and 34 (11.8%) bla_{CMY}. WGS of β-lactamase negative or carbapenem-resistant isolates identified uncommon ESBLs and carbapenemases, including bla_{NDM} and bla_{IMP}, and confirmed all PCR-positive genotypes. No evidence of transmission among intensive care unit patients was identified. We demonstrate that our PCR assays enable the rapid and cost-effective identification of ESBLs in the hospital setting, which has important infection control and therapeutic implications.

Introduction

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

AMR in 3GC-R Enterobacteriaceae is generally conferred by the presence of an ESBL or the over-expression of a chromosomal or plasmid-borne AmpC β-lactamase; however, the prevalence of these determinants vary greatly depending on the geographic region under examination [5]. CTX-M-type ESBLs are currently the most common AMR determinants found in 3GC-R Enterobacteriaceae isolated in Australian hospitals, with CTX-M-1 and CTX-M-9 groups collectively comprising 86.2% of identified CTX-M-type enzymes [4]. Detection of ESBL subtypes is not standard clinical practice; instead, their presence is typically inferred from antimicrobial susceptibility patterns and phenotypic confirmatory tests, which are laborious, costly (~$AUD25/isolate), and time-consuming (>24h) to perform [6]. Use of rapid, accurate, and inexpensive molecular-based diagnostics for AMR determinant detection in the clinical setting may improve patient outcomes by enabling more rapid, directed, and personalized treatment strategies, thereby improving antibiotic stewardship measures and

76 reducing AMR burden. This utility has already been demonstrated in a number of clinical
77 settings including for bloodstream infections, pneumonia, and infection control screening [7-
78 9].
79
80 The implementation of whole-genome sequencing (WGS) into clinical practice is rapidly
81 becoming a reality in many parts of the world. In the infectious diseases field, WGS enables
82 the precise identification of AMR determinants in clinical samples (including polymicrobial
83 specimens), and the inference of population structure and transmission dynamics on a local
84 (e.g. single hospital) through to a global scale. Multiple studies have used WGS to better
85 understand Enterobacteriaceae population structure in the hospital setting [10, 11], often in
86 an effort to better understand transmission dynamics and to control nosocomial infection
87 spread. These studies have provided valuable insights into the population structure of the
88 Enterobacteriaceae, including the identification of a rapid clonal expansion of *E. coli*
89 sequence type 131 (ST131) [12, 13] and an increased appreciation for the high rates of AMR
90 in *K. pneumoniae*, which is now recognized as a significant global threat [3].
91
92 In the current study, we prospectively collected all 3GC-R Enterobacteriaceae identified over
93 a 14-month period at the Sunshine Coast University Hospital, Queensland, Australia, a 450-
94 bed, regional teaching and tertiary hospital. Using large-scale comparative genomics, we
95 developed a novel pentaplex assay and a bla_{CMY} singleplex assay to rapidly detect the most
96 commonly identified β-lactamase (including ESBL) genes in 3GC-R Enterobacteriaceae. In
97 association with WGS, we used our assays to determine the molecular epidemiology of the
98 β-lactamase genes in our sample set and compared the accuracy of our assays to both
99 genotypic and phenotypic typing methods to validate accuracy, sensitivity and specificity.
100 Additionally, we determined the population structure and transmission dynamics of our

101 3GC-R Enterobacteriaceae population to identify potential cases of nosocomial spread or

102 outbreak clusters.

103 **Methods**

104

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

108

109 **Sample collection and clinical information.** Clinical specimens from 258 adult and pediatric
110 patients culturing 3GC-R Enterobacteriaceae were collected prospectively from July 2017
111 through September 2018 at the Sunshine Coast University Hospital microbiology laboratory
112 (*n*=288). Our patient cohort was consistent with other studies, being predominantly female
113 (64.0%) and aged over 65 (60.9%), with an age range of 2 to 98 years old. Intensive care unit
114 (ICU) patient specimens comprised 54 (18.8%) of the total collection, and were obtained
115 from 48 ICU patients over a 12-month period. From the ICU cases, 37 *E. coli*, 9 *K.*
116 *pneumoniae,* two *K. oxytoca* and one *E. cloacae* were identified from rectal swabs (*n*=49;
117 90.7%) with urine, sputum and pus making up the remaining five samples. Of the non-ICU
118 patients (*n*=216 with 234 samples), the breakdown of clinical samples included urine (*n*=154;
119 65.8%), rectal swabs (*n*=66; 28.2%), blood (*n*=7; 3%) and pus (*n*=6; 2.6%). Isolates were
120 stored in Luria Bertani broth containing 20% glycerol at -80°C. Patient and hospital
121 epidemiological data were also documented and linked to each specimen. Once collected,
122 isolates were speciated using the VITEK® 2 MALDI-TOF MS with the GN ID card (bioMérieux,
123 Murrarie, QLD, Australia). Isolate information is detailed in Table S1.

124

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

129

130 **DNA preparation.** For PCR, crude DNA was extracted from all isolates using 5% chelex-100
131 resin (Bio-Rad Laboratories, Gladesville, NSW, Australia) in 96-well plate format. Due to the
132 potential for cross-contamination, approximately six no-template controls were randomly
133 included on each DNA plate. A small toothpick-sized scraping of culture was suspended into
134 the chelex resin, followed by plate sealing and heating to 95°C for 10 min in a PCR
135 thermocycler. Following centrifugation at 4,000 x *g* for 2 min, a 1:10 dilution of DNA was
136 prepared in molecular-grade H_2O (Sigma-Aldrich, Castle Hill, NSW, Australia); this dilution
137 was used for PCR. For a subset of samples (*n*=50), a pinhead-sized amount of culture
138 collected using a sterile 10 µL pipette tip was placed directly into 4 µL of PCR mastermix to
139 facilitate a more rapid turnaround time. For WGS, DNA was extracted using the Quick-DNA
140 miniprep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions.

141

142 **PCR development and β-lactamase detection.** A pentaplex real-time PCR assay was
143 developed to detect the two major CTX-M ESBL groups ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$), bla_{SHV} and
144 bla_{TEM} (designed to amplify both ESBL and non-ESBL bla_{SHV} and bla_{TEM}), and all variants of
145 bla_{CMY} β-lactamase genes (Figure 1). To identify conserved regions for assay design, all
146 publicly available genetic sequences for $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{CMY}, bla_{SHV}, and bla_{TEM} were
147 downloaded from the NCBI Nucleotide database (as at March 2019) and aligned using
148 Clustal Omega v1.2.2. Aligned sequences were visualized in Geneious v11.0.4. Candidate
149 primers and probes were identified in Primer Express v3.0 (Applied Biosystems, Foster City,
150 California, USA) or Primer3 v0.4.0 [14] followed by primer dimer and heterodimer
151 assessment using NetPrimer (http://www.premierbiosoft.com/netprimer/).

152

153 For the pentaplex assay, Black Hole Quencher probes were used to enable multiplexing of
154 five assays in a single PCR well. The following primers and probes (5' to 3'; Biosearch

155 Technologies, Novato, CA, USA; Macrogen Inc., Geumcheon-gu, Seoul, Republic of Korea)
156 were designed: CTX-1 (CTX1_F: CGAMACGTTCCGTCTCGAC, CTX1_R:
157 CTTTCATCCAYGTCACCAGCTG, CTX1_P1: FAM-CGTGATACCACTTCACCTCGGG-BHQ1), CTX-9
158 (CTX9_F: CGTCSCGCTCATCGATACC, CTX9_R: CAGCTGCTTTTGCGTTTCACTC, CTX9_P1: HEX-
159 CGCTTTCCAATGTGCAGTACC-BHQ1), SHV (SHV_F: CCARCGTCTGAGCGCC, SHV_R:
160 TATCGGCGATAAACCAGCCC, SHV_P1: Cy5-CAGCACGGAGCGGATCA-BHQ2), TEM (TEM_F:
161 TGTGGYGCGGTATTATCCCGT, TEM_R: TGCATAATTCTCTTACTGTCAWGCCA, TEM_P1: Cy5.5-
162 CACCAGTCACAGAAAAGCATCT-BHQ2), and a previously described 16S assay [15] using a
163 different fluorophore on the probe (Texas Red-CACGAGCTGACGACARCCATGCA-BHQ2) to
164 accommodate a pentaplex format. Last, an additional assay targeting CMY was included as a
165 singleplex assay: CMY (CMY_F: TGGCGTATTGGYGATATGTA, CMY_R:
166 TTATGCACCCATGAGGCTTT, CMY_Pr: FAM-TGGGAGATGCTGAACTGGCC-BHQ1).
167 Coincidentally, the reverse primer sequence for our CMY assay was identical to one designed
168 previously [16].
169
170 PCRs were carried out using 0.1 µM of the 16S probe, 0.2 µM of the 16S primers, TEM
171 primers and the CTX-1, CTX-9, and TEM probes, 0.3 µM of the SHV primers, and 0.4 µM of
172 the CTX-1, CTX-9, and CMY primers, and the SHV and CMY probes. Reactions contained 1X
173 Sso Advanced Universal Probes Supermix (Bio-Rad), primers, probes, 1 µL DNA template,
174 and molecular grade H_2O to a 5 µL total reaction volume. PCRs were performed on a CFX96
175 Touch Real-Time PCR Detection System using white Hard Shell 96-well PCR plates and
176 Microseal 'B' Adhesive seals (Bio-Rad) or 0.2 mL 8-tube PCR strips (Bio-Rad). Thermocycling
177 conditions comprised an initial 2 min denaturation at 95 °C, followed by 45 cycles of 95 °C
178 for 1 sec and 60 °C for 3 sec (total run time of 39 min). Due to the inclusion of the 16S rDNA
179 internal control, each sample were run as a single reaction, with any PCR failures according
180 to 16S (either due to no amplification, or amplification >25 cycles) repeated as required.

181

182 **Whole-genome sequencing (WGS).** WGS was performed at the Australian Centre of
183 Ecogenomics (University of Queensland, St Lucia, QLD, Australia) using the metagenomics
184 sequencing protocol on the NextSeq 500 instrument (Illumina, San Diego, CA, USA) with
185 libraries constructed using Nextera DNA Flex (Illumina).

186

187 **Genomic analysis.** All genomic data were lightly quality-filtered using Trimmomatic v0.39
188 [17] using parameters described elsewhere [18]. Following quality filtering, genomes for
189 each sample were screened for the presence of multiple species using Kraken 2 v2.0.8 [19].
190 Single-species genomes were assembled using MGAP v1.1
191 (https://github.com/dsarov/MGAP---Microbial-Genome-Assembler-Pipeline), whereas
192 genomes containing multiple species were assembled with metaSPAdes [20]. Assembled
193 contigs were binned with MaxBin v2.0 [21] and speciated with Kraken 2 v2.0.8 [19].
194 Antibiotic resistance genes were identified through the Resistance Gene Identifier tool
195 within the Comprehensive Antimicrobial Resistance Database (CARD) [22]. Phylogenomic
196 analysis was performed on biallelic, orthologous, core-genome single-nucleotide
197 polymorphisms (SNPs) using SPANDx v3.2.1 [23]. Samples identified as containing multiple
198 strains according to the method described by Aziz and colleagues [24] were excluded from
199 phylogenetic analysis (*n*=7). Samples containing multiple species were split using the
200 procedure described above and included in phylogenetic analysis (*n*=4). *E. coli* O157:H7 Sakai
201 [25] or *K. pneumoniae* HS11286 [26] were used as read mapping reference genomes for
202 species-specific analyses. To achieve the highest possible resolution, error-corrected
203 assemblies of strains with matching multilocus sequence types (STs) were used as reference
204 genomes for transmission analyses. ST profiles were determined using SRST2 v0.2.0 [27].
205 Phylogenetic trees were reconstructed using the heuristic search feature of PAUP v4.4.7 [28]
206 and visualized in iTOL v5.5[29].

207

208 **WGS data.** Raw sequence reads are available on the NCBI Sequence Read Archive database

209 under BioProject accession number PRJNA606985.

210 **Results and Discussion**

211 ESBL enzymes have established themselves as major contributors of AMR in Gram-negative
212 bacteria and are increasing in prevalence, particularly in the community setting. Over the
213 course of 14 months, we collected isolates from all diagnosed cases of 3GC-R
214 Enterobacteriaceae in a regional hospital in Southeast Queensland, Australia. Among the
215 288 Gram-negative isolates collected during this study, *E. coli* and *K. pneumoniae* were
216 dominant, comprising 80.2% and 17.0% of the isolates, respectively (Table 1). The remaining
217 isolates were identified as *E. cloacae* (1.4%; *n*=4), *K. oxytoca* (1.0%; *n*=3), and *K. aerogenes*
218 (0.3%; *n*=1). Resistance to ceftriaxone, ceftazidime and cefepime was common and observed
219 in 96.2%, 93.4%, and 86.8% of isolates, respectively, whereas cefoxitin resistance was noted
220 in only 81 (28.1%) isolates (Table 1). Meropenem resistance was seldom observed, with only
221 six strains (2.1%) exhibiting resistance to this carbapenem. The double disc diffusion method
222 for ESBL phenotypic testing was negative in 32 (11.1%) isolates, indicating the presence of a
223 3GC-R mechanism with retention of a resistant phenotype in the presence of a β-lactamase
224 inhibitor (e.g. clavulanic acid), likely due to over-expression of an AmpC β-lactamase. The
225 remaining 256 (88.9%) isolates were positive according to the double disc diffusion method,
226 exhibiting sensitivity in the presence of a β-lactamase inhibitor.

227

228 *Redesign of current ESBL PCR assays to improve specificity and detection rates.* We next set
229 out to identify the abundance of 3GC-R AMR determinants in our isolate collection using
230 real-time PCR. The rapid expansion of WGS data in recent years has enabled improvements
231 to be made upon assays designed in the pre-WGS era, which risk being either non-specific or
232 intolerant to sequence diversity within enzyme families. Given a sufficiently large and
233 diverse dataset, sequence diversity can result in a non-trivial proportion of false-negative
234 calls, skewing epidemiological observations. Examination of previously published ESBL assays
235 [30] identified several mismatches in primer/probe pairs, with the mismatches especially

236 problematic for the CTX-M enzymes (data not shown). To overcome issues with false-
237 negative results using existing assays, we analyzed the extensive volume of publicly available
238 sequence data to identify conserved sequence regions suitable for assay design. Our
239 redesigned assays target the genes encoding the most common ESBLs (CTX-M group 1, CTX-
240 M group 9), along with SHV and TEM β-lactamases, which can evolve to become ESBLs
241 through mutations in their active sites or contribute to clinically relevant AMR due to
242 increased expression.
243
244 *ESBL and β-lactamase prevalence in 3GC-R Enterobacteriaceae.* Preliminary validation of our
245 newly designed PCR assays was performed to determine their accuracy and specificity. The
246 assays were first tested on a characterized set of 12 strains (Table S2) that have previously
247 been subjected to WGS [31]. Our assays showed 100% sensitivity and specificity for all
248 enzymes (CTX-M group 1, CTX-M group 9, CMY, SHV and TEM) according to corresponding
249 WGS data. Following this confirmation, our assays were run across the entire isolate set
250 (*n*=288) in pentaplex format ($bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-9}}$ group, bla_{SHV}, bla_{TEM}, 16S rDNA; Figure
251 1), with bla_{CMY} tested in singleplex format. Our assays performed well, with 100%
252 concordance between PCR genotypes and WGS of 98 isolates, and no false-negatives
253 identified. Overall, a β-lactamase genotype was identified in 277 (96.2%) isolates using PCR.
254 Consistent with the previously reported prevalence of 3GC-R enzymes in Australia and New
255 Zealand [10], we found that $bla_{\text{CTX-M-1}}$ group was most common, being present in 144/288
256 (50%) isolates, including 105/231 (45.5%) *E. coli* and 36/49 (73.5%) *K. pneumoniae* isolates.
257 The $bla_{\text{CTX-M-9}}$ group was next most common, being found in 93/288 (32.3%) isolates,
258 including 89/231 (38.5%) *E. coli* and 4/49 (8.2%) *K. pneumoniae* isolates. The presence of
259 both $bla_{\text{CTX-M-1}}$ group and $bla_{\text{CTX-M-9}}$ group genes was identified in five (1.7%) *E. coli* and one *K.*
260 *pneumoniae* isolate. Of the eight isolates belonging to other species, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$
261 group genes were present in 4/8 (50%) and 0/8 (0%) of these isolates, respectively. The

262 acquired *ampC* gene, bla_{CMY}, was identified in 30/231 (13%) of *E. coli*, 3/49 (6.1%) of *K.*
263 *pneumoniae*, and 1/8 (13%) other Enterobacteriaceae species. bla_{CMY} and $bla_{CTX\text{-}M\text{-}1}$ were both
264 present in seven isolates (five *E. coli*, one *K. pneumoniae*, and one *E. cloacae*) and bla_{CMY} and
265 $bla_{CTX\text{-}M\text{-}9}$ were present in two *E. coli* isolates only (Table 1).
266
267 Seven strains (five *E. coli* and two *K. pneumoniae*) have bla_{SHV} only; nine *E. coli* have bla_{TEM}
268 only, and seven (five *K. pneumoniae*, one *E. coli*, and one *E. cloacae*) have both bla_{SHV} and
269 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
270 typically cause 3GC-R, their presence in strains that lack CTX-M-type ESBLs should be
271 investigated, as these enzymes can occasionally confer 3GC-R through active site mutation
272 or overexpression [5]. Of the 14 strains encoding bla_{SHV} but not $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$
273 group, or bla_{CMY} enzymes, five had accompanying WGS data, which permitted assessment of
274 bla_{SHV} ESBL status. In all cases, the bla_{SHV} variant was an ESBL (two $bla_{SHV\text{-}2}$, one $bla_{SHV\text{-}64}$, one
275 $bla_{SHV\text{-}66}$, and one $bla_{SHV\text{-}134}$) [32]. In contrast, none of the four strains encoding bla_{TEM} but not
276 $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CMY}, or ESBL-encoding bla_{SHV} enzymes were ESBLs, with all
277 being $bla_{TEM\text{-}1}$. Three of these were negative with the double disc diffusion method,
278 indicating probable chromosomal mutations underpinning this phenotype. The basis for the
279 ESBL phenotype in the fourth strain could not be elucidated.
280
281 Due to the conserved nature of our TEM and SHV assays to detect all enzyme variants, we
282 do not recommend these assays for identifying ESBL-conferring mutations in bla_{TEM} or bla_{SHV};
283 rather, they can only be used to guide further diagnostic tests. However, the quantitative
284 nature of our pentaplex assay, which includes an internal 16S rDNA control, permits the
285 detection of copy number increases in the β-lactamase genes. By examining the cycles-to
286 threshold differences between 16S and bla_{TEM} and bla_{SHV}, we identified an ~8-10x copy
287 number increase in SCHI0016.S.139, which also possessed $bla_{CTX\text{-}M\text{-}15}$. Consistent with this

288 PCR result, WGS verified that this strain harbored a multicopy plasmid carrying *bla*$_{TEM-135}$.
289 TEM-135 differs from TEM-1 by having a M182T mutation that confers improved β -
290 lactamase stability and potential for third-generation cephalosporin hydrolysis and ESBL
291 activity [33]. Therefore, examining β-lactamase copy number is important when determining
292 ESBL potential from genetic or genomic data, particularly in strains where common ESBLs
293 are not identified.
294
295 *Correlation of bla*$_{CMY}$ *and cefoxitin resistance*. Of the 81 isolates resistant to cefoxitin by
296 phenotypic methods, *bla*$_{CMY}$ was detected in only 31 (38.3%) cases. Resistance to cefoxitin is
297 often used as a surrogate marker for AmpC presence, although this approach is imperfect
298 [34]. This correlation is also dependent on the MIC, with higher MICs more reliably
299 predicting AmpC β-lactamase presence [34]. Our findings concur with this prior work, with
300 61.7% of isolates failing to amplify the *bla*$_{CMY}$ target, suggesting a lack of sensitivity in using
301 cefoxitin as a screening tool for AmpC enzymes. In support of this hypothesis, AmpC has
302 previously been reported as an underappreciated mechanism of 3GC-R due to a lack of
303 standardized testing for its detection [35]. As such, molecular methods such as PCR that can
304 detect other common and emerging acquired *ampC* gene families (e.g. *bla*$_{DHA}$, *bla*$_{OXY}$, *bla*$_{MIR}$)
305 may prove useful for infection control management and treatment of multidrug-resistant
306 Gram-negative infections. This is particularly true in the case of piperacillin-tazobactam,
307 which may not be a stable or reliable antibiotic against AmpC producers [10, 36].
308
309 *Whole-genome sequencing analysis identifies uncommon ESBL genes.* Eleven (3.8%) isolates
310 negative with all PCR assays except the 16S rDNA internal control (seven *E. coli*, one *K.*
311 *pneumoniae*, one *K. oxytoca*, one *K. aerogenes* and one *E. cloacae*) were subjected to WGS
312 to determine the molecular basis for their 3GC-R phenotype. Six were found to carry
313 chromosomal AmpC enzymes, with three *E. coli* encoding *bla*$_{DHA-1}$, one *K. pneumoniae/K.*

314 *oxytoca* mixture harboring *bla*$_{OXY-2-4}$, one *K. oxytoca* carrying *bla*$_{OXY-2-1}$, and one *E. cloacae*
315 carrying *bla*$_{ACT-17}$. The remaining five isolates had no discernable 3GC-R determinants,
316 although misclassification via automated susceptibility testing or upregulation of
317 chromosomal AmpC β-lactamases are both possible causes for this discrepancy.
318
319 Six isolates (three *E. cloacae*, two *E. coli* and one *K. pneumoniae*) exhibiting meropenem
320 resistance alongside 3GC-R were also subjected to WGS to determine the basis of resistance
321 towards this carbapenem. As expected, meropenem resistance was mostly conferred by
322 carbapenemases, with 4/6 (66.6%) isolates possessing one of these enzymes. *bla*$_{NDM-1}$ or
323 *bla*$_{IMP-4}$ were identified in two *E. cloacae* isolates, *bla*$_{NDM-5}$ was identified in an *E. coli* strain,
324 and an OXA-48-like enzyme, *bla*$_{OXA-232}$, was identified in a *K. pneumoniae* isolate. No
325 carbapenemase gene could be identified in the remaining two isolates (one *E. coli* and one *E.*
326 *cloacae*); although not investigated in this study, chromosomally-encoded porin loss or
327 efflux pump overexpression may form the basis of resistance in these strains.
328
329 *Monitoring patient-to-patient spread in an intensive care unit.* Fifty-four (18.8%) 3GC-R
330 isolates obtained during this study originated from ICU-admitted patients. Resistance to
331 ceftriaxone and cefoxitin was identified in 100% and 31.4% of isolates, respectively, and
332 meropenem resistance was identified in three (5.5%) isolates. Due to their dominance, only
333 *E. coli* and *K. pneumoniae* genomes were analyzed to assess for potential transmission
334 events. Among the *E. coli* population (Figure 2), isolates SCHI0016.S.60 and SCHI0016.S.62,
335 and SCHI0016.S.265 and SCHI0016.S.46, were separated by 10 SNPs and 2 SNPs,
336 respectively; however, both pairs came from individual patients, reflecting expected levels of
337 within-host evolution of a single infecting strain. *E. coli* isolates SCHI0016.S.42 and
338 SCHI0016.S.294 were very similar, being separated by 13 SNPs. Although both strains were
339 isolated from ICU admitted patients, SCHI0016.S.294 was isolated 306 days after

340 SCHI0016.S.42 with no other linking epidemiology identified. Additionally, no similar strains
341 were isolated from ICU patients over this time period suggesting that these do not represent
342 transmission within the ICU. *E. coli* isolates SCHI0016.S.172 and SCHI0016.S.274 differed by
343 approximately 300 SNPs, with no epidemiological or temporal correlation between patients.
344 A similar number of SNPs separated isolates SCHI0016.S.112 and SCHI0016.S.229, again with
345 no demonstrable epidemiological link. Among the *K. pneumoniae* ICU strains (Figure 3), the
346 closest isolates, SCHI0016.S.15 and SCHI0016.S.261, differed by 361 SNPs, and again had no
347 epidemiological link. All patients in our ICU, and all patients in other hospital wards that are
348 infected or colonized with confirmed ESBL-producing *E. coli* and *K. pneumoniae*, have a
349 single room with an assigned nurse (1:1 ratio), with contact precautions exercised as
350 standard practice. In additional, hand hygiene rates in our hospital ICU are generally above
351 80% (Australian national benchmark) [37]. Taken together, our results demonstrate no
352 evidence of ESBL transmission in the ICU ward over the study period, suggesting the
353 implementation of effective hygiene measures by ICU staff.
354
355 *Population structure of E. coli and K. pneumoniae genomes.* We subjected an additional 36
356 strains to WGS (total *n*=98) to capture a snapshot of genomic diversity and population
357 structure in our samples (Figures 2 and 3). *In silico* MLST of the 79 genome-sequenced *E. coli*
358 strains found that ST131 was most common ST (*n*=11; 13.9%). This finding is consistent with
359 a recent study of the population structure of Australian, New Zealand, and Singaporean
360 multidrug-resistant *E. coli* isolates, where ST131 was identified as the dominant strain [10].
361 The highly successful ST131 clone has spread worldwide, where it causes urinary tract and
362 bloodstream infections, particularly community-associated infections [38]. Overall,
363 depending on the country, ST131 accounts for 12–27% of all community-acquired *E. coli*
364 infections [39]. In addition, transmission events have been documented among hospitalized
365 patients, although person-to-person spread appears to be more important between

366 household contacts [40]. Among our ST131 isolates, we found high frequencies of
367 fluoroquinolone resistance-conferring variants ($GyrA_{S83L}$, $GyrA_{D87N}$, and $ParC_{S80I}$) alongside
368 frequent carriage of plasmid-borne CTX-M-14 (CTX-M-9 group) and CTX-M-15 (CTX-M-9
369 group) ESBLs. We also identified three strains belonging to the emerging multi-drug resistant
370 pandemic clone, ST1193: SCHI0016.S.133, SCHI0016.S.172 and SCHI0016.S.274, although the
371 latter strain was excluded from the phylogenetic analysis due to the presence of mixtures.
372 These isolates also possessed the same three fluoroquinolone-resistant SNPs as the ST131
373 strains. It has been proposed that ST1193 strains evolved fluoroquinolone resistance
374 relatively recently through multiple recombination events with other AMR *E. coli* strains
375 [41].
376
377 Phylogenomic analysis of our 10 *K. pneumoniae* genomes identified several distinct lineages,
378 which comprised two ST14 strains and one each of ST17, ST307, ST309, ST395, ST656, ST697,
379 ST873, and ST969. Of note, the hypervirulent and globally disseminated ST258 clone, which
380 is responsible for a large number of hospital deaths [42], was not identified in our dataset.
381 However, we identified another globally disseminated clone, ST395, which has also been
382 retrieved from clinical cases in Europe and Asia [43]. Concerningly, this ST395 isolate,
383 SCHI0016.S.137, carried both the ESBL genes bla_{SHV-11} and $bla_{CTX-M-15}$ and the OXA-48-like
384 carbapenemase, $bla_{OXA-232}$, the latter of which confers low-level meropenem resistance.
385
386 This study had several recognized limitations. First, isolates were only collected from a
387 hospital setting rather than from both nosocomial and community sources. It is well-
388 established that differences in AMR patterns and their associated molecular mechanisms
389 exist between hospital and community settings. Second, the absence of certain high-risk
390 populations within our hospital (e.g. solid organ or hematopoietic stem cell transplantation,
391 burns) limits the generalizability of our findings to other settings. Third, our PCR assays can

392 only identify the presence or change in copy number of the most common ESBL and *ampC*
393 genes. Although these loci make up the majority (>90%) of β-lactamase enzymes present in
394 3GC-R Enterobacteriaceae, they do not capture all potential ESBLs, leaving less common
395 ESBL and *ampC* genes unidentified. In addition, PCR assays for bla_{TEM} or bla_{SHV} cannot
396 discriminate between ESBLs and non-ESBLs, with additional assays or concurrent WGS
397 required to confirm ESBL status. Our assays also cannot detect chromosomal changes
398 leading to AMR (e.g. porin mutations or efflux pump up-regulation), making these assays less
399 useful for 3GC-R bacteria that frequently encode these AMR determinants (e.g.
400 *Pseudomonas aeruginosa*). One way to address detection issues is to undertake WGS on all
401 samples; however, as demonstrated in this study, some AMR determinants will likely remain
402 cryptic due to their novelty, thereby requiring additional functional work to verify their
403 causative nature. Fourth, our PCR was unable to distinguish whether β-lactamase genes
404 were present on low-copy plasmids or were chromosomally incorporated, which has
405 implications for AMR transmissibility between and among bacterial populations. Finally,
406 ~40% of samples were obtained from rectal swabs, which have inherent limitations in
407 detection of multidrug-resistant organisms due to the high microbial burden from this
408 specimen type coupled with difficulties in adequate sample collection. It is therefore likely
409 that we did not capture all 3GC-R clinical isolates during our study period due to the
410 inherent difficulties associated with this sampling technique, and thus may have missed
411 potential transmission events in the hospital.
412

413 **Conclusion**

414

415 Taken together, our findings identified a predominance of CTX-M (and to a lesser extent,
416 CMY) β-lactamases among 3GC-R Enterobacteriaceae in the Sunshine Coast region. These
417 findings are consistent with global findings of Gram-negative bacterial AMR determinants,
418 particularly among *E. coli*. Our study demonstrates the likely absence of 3GC-R
419 Enterobacteriaceae transmission over a one-year period in an ICU, indicating effective
420 infection control processes for these pathogens. We show that rapid molecular assays, such
421 as our multiplex PCR, can detect common β-lactamase genes in a clinical setting within 40
422 minutes, including directly from bacterial culture. Further research is required to determine
423 whether our molecular assays can be used directly on clinical specimens, and whether the
424 implementation of molecular assays and more advanced technologies (e.g. WGS) is
425 associated with improved patient outcomes in the hospital setting.

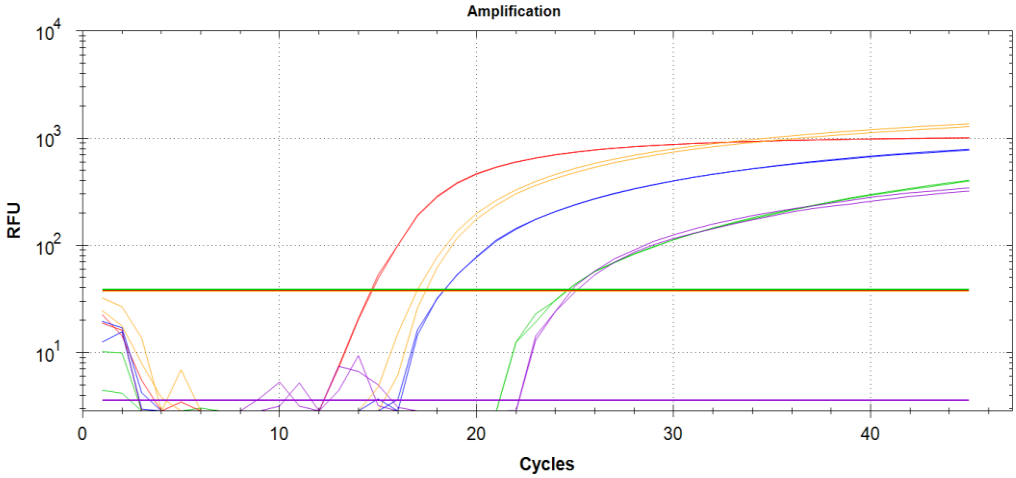
426

427 **Acknowledgements**

428

429 This study was funded by the Wishlist Sunshine Coast Health Foundation (awarded to AGS,
430 KC, SS, and DSS). DSS and EPP were funded by Advance Queensland (grants AQRF13016-
431 17RD2 and AQIRF0362018, respectively). We thank Dr Delaney Burnard and the Sunshine
432 Coast University Hospital pathology laboratory for assistance.

433 **Figures**



434

435 **Figure 1: Pentaplex PCR performance on third-generation cephalosporin-resistant**

436 **Enterobacteriaceae.** Red, 16S rDNA gene; orange, CTX-M group 9; blue, TEM; green, CTX-M

437 group 1; purple, SHV.

438

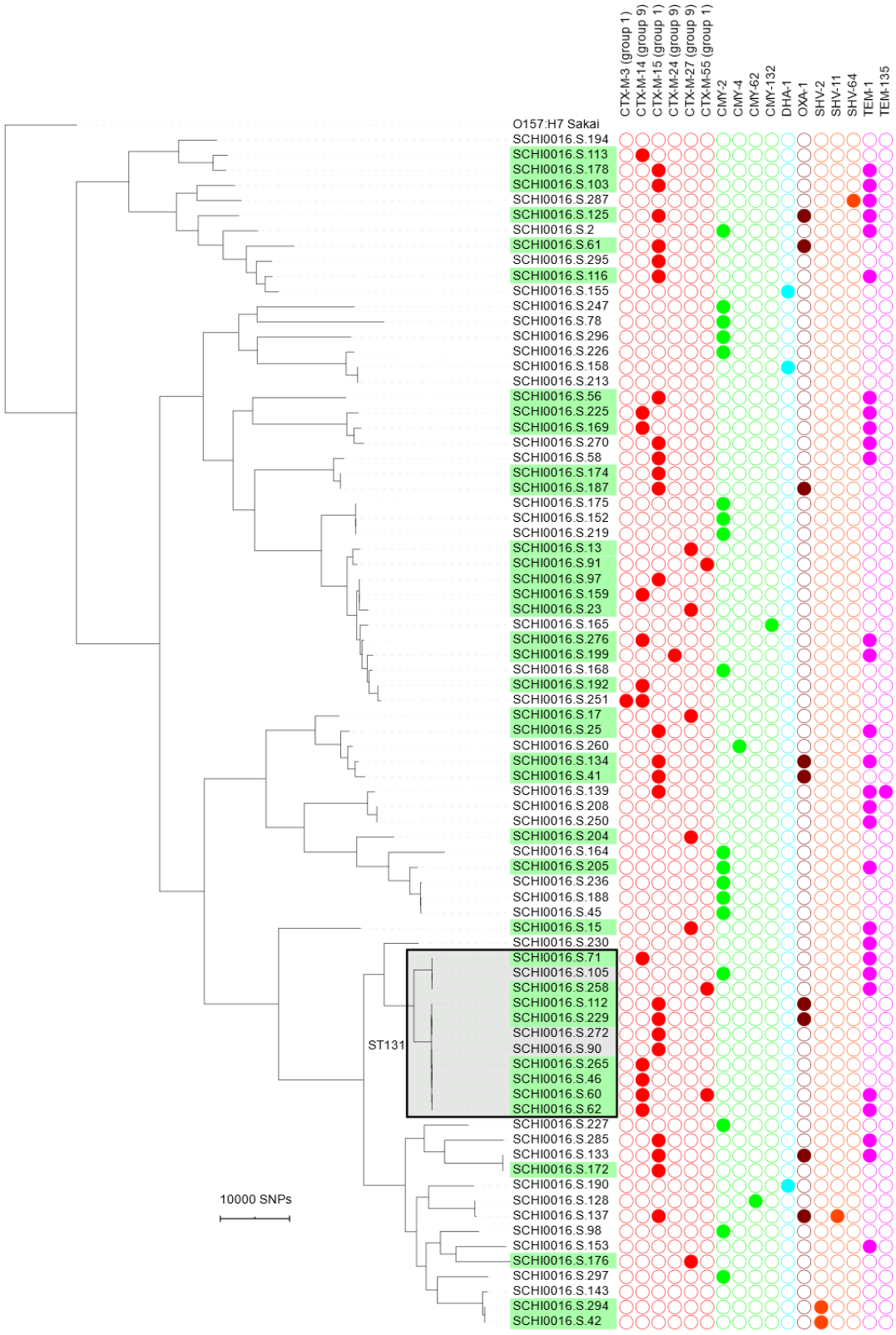


Figure 2. Whole-genome maximum parsimony phylogeny of 79 third-generation cephalosporin-resistant *Escherichia coli* isolates and their β-lactamase gene complement. Sequence type (ST)131 is indicated by the gray-shaded box; strains isolated from intensive care unit patients are highlighted in green.

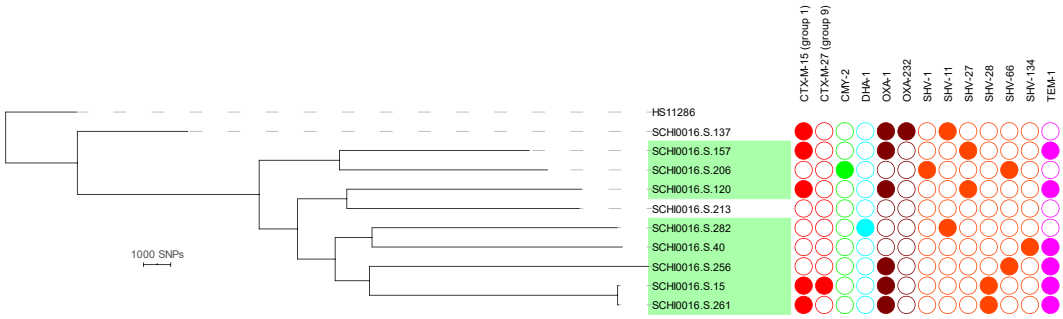


Figure 3. Whole-genome phylogeny and presence of β-lactamase enzymes in the *Klebsiella pneumoniae strains* isolated in our study. Isolates shaded in green were isolated from the intensive care unit.

450 **References**

451 1. Gupta, V., et al., *National prevalence estimates for resistant*
452 *Enterobacteriaceae and Acinetobacter species in hospitalized patients in*
453 *the United States.* Int J Infect Dis, 2019.
454 2. Biehl, L.M., et al., *Colonization and infection with extended spectrum beta-*
455 *lactamase producing Enterobacteriaceae in high-risk patients - Review of*
456 *the literature from a clinical perspective.* Crit Rev Microbiol, 2016. **42**(1):
457 p. 1-16.
458 3. *Antimicrobial resistance - global report on surveillance 2014*. 2014;
459 Available from:
460 https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564
461 748_eng.pdf;jsessionid=519244EE0EEF520027CE4098504150B4?seque
462 nce=1.
463 4. *Gram-negative Sepsis Outcome Program 2018 Report*. 2018, Australian
464 Group on Antimicrobial Resistance p. 27-31.
465 5. Paterson, D.L. and R.A. Bonomo, *Extended-spectrum beta-lactamases: a*
466 *clinical update.* Clin Microbiol Rev, 2005. **18**(4): p. 657-86.
467 6. Consortium, B.a.B. *Technology overview | BD MAX Enteric Bacterial Panel*
468 *for identifying pathogens in contagious gastroenteritis | Advice | NICE*.
469 2015 [cited 2020 14-2-2020]; Available from:
470 https://www.nice.org.uk/advice/mib32/chapter/Technology-overview.
471 7. MacVane, S.H. and F.S. Nolte, *Benefits of Adding a Rapid PCR-Based Blood*
472 *Culture Identification Panel to an Established Antimicrobial Stewardship*
473 *Program.* J Clin Microbiol, 2016. **54**(10): p. 2455-63.
474 8. Echavarria, M., et al., *Clinical impact of rapid molecular detection of*
475 *respiratory pathogens in patients with acute respiratory infection.* J Clin
476 Virol, 2018. **108**: p. 90-95.
477 9. Souverein, D., et al., *Clinical sensitivity and specificity of the Check-Points*
478 *Check-Direct ESBL Screen for BD MAX, a real-time PCR for direct ESBL*
479 *detection from rectal swabs.* J Antimicrob Chemother, 2017. **72**(9): p.
480 2512-2518.
481 10. Harris, P.N.A., et al., *Whole genome analysis of cephalosporin-resistant*
482 *Escherichia coli from bloodstream infections in Australia, New Zealand and*
483 *Singapore: high prevalence of CMY-2 producers and ST131 carrying*
484 *blaCTX-M-15 and blaCTX-M-27.* J Antimicrob Chemother, 2018. **73**(3): p.
485 634-642.
486 11. Long, S.W., et al., *Population Genomic Analysis of 1,777 Extended-Spectrum*
487 *Beta-Lactamase-Producing Klebsiella pneumoniae Isolates, Houston, Texas:*
488 *Unexpected Abundance of Clonal Group 307.* mBio, 2017. **8**(3).
489 12. Petty, N.K., et al., *Global dissemination of a multidrug resistant Escherichia*
490 *coli clone.* Proc Natl Acad Sci U S A, 2014. **111**(15): p. 5694-9.
491 13. Johnson, J.R., et al., *Escherichia coli sequence type ST131 as the major cause*
492 *of serious multidrug-resistant E. coli infections in the United States.* Clin
493 Infect Dis, 2010. **51**(3): p. 286-94.
494 14. Untergasser, A., et al., *Primer3--new capabilities and interfaces.* Nucleic
495 Acids Res, 2012. **40**(15): p. e115.

496 15. Yang, S., et al., *Quantitative multiprobe PCR assay for simultaneous*
497 *detection and identification to species level of bacterial pathogens.* J Clin
498 Microbiol, 2002. **40**(9): p. 3449-54.
499 16. Bae, I.K., et al., *Detection of Carbapenemases in Clinical Enterobacteriaceae*
500 *Isolates Using the VITEK AST-N202 Card.* Infect Chemother, 2015. **47**(3): p.
501 167-74.
502 17. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for*
503 *Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-20.
504 18. Price, E.P., et al., *Transcriptomic analysis of longitudinal Burkholderia*
505 *pseudomallei infecting the cystic fibrosis lung.* Microb Genom, 2018. **4**(8).
506 19. Wood, D.E., J. Lu, and B. Langmead, *Improved metagenomic analysis with*
507 *Kraken 2.* Genome Biol, 2019. **20**(1): p. 257.
508 20. Nurk, S., et al., *metaSPAdes: a new versatile metagenomic assembler.*
509 Genome Res, 2017. **27**(5): p. 824-834.
510 21. Wu, Y.W., B.A. Simmons, and S.W. Singer, *MaxBin 2.0: an automated*
511 *binning algorithm to recover genomes from multiple metagenomic datasets.*
512 Bioinformatics, 2016. **32**(4): p. 605-7.
513 22. Jia, B., et al., *CARD 2017: expansion and model-centric curation of the*
514 *comprehensive antibiotic resistance database.* Nucleic Acids Res, 2017.
515 **45**(D1): p. D566-D573.
516 23. Sarovich, D.S. and E.P. Price, *SPANDx: A genomics pipeline for comparative*
517 *analysis of large haploid whole genome re-sequencing datasets.* BMC Res
518 Notes, 2014. **7**: p. 618.
519 24. Aziz, A., et al., *Comparative genomics confirms a rare melioidosis human-to-*
520 *human transmission event and reveals incorrect phylogenomic*
521 *reconstruction due to polyclonality.* Microb Genom, 2020.
522 25. Hayashi, T., et al., *Complete genome sequence of enterohemorrhagic*
523 *Escherichia coli O157:H7 and genomic comparison with a laboratory strain*
524 *K-12.* DNA Res, 2001. **8**(1): p. 11-22.
525 26. Liu, P., et al., *Complete genome sequence of Klebsiella pneumoniae subsp.*
526 *pneumoniae HS11286, a multidrug-resistant strain isolated from human*
527 *sputum.* J Bacteriol, 2012. **194**(7): p. 1841-2.
528 27. Inouye, M., et al., *SRST2: Rapid genomic surveillance for public health and*
529 *hospital microbiology labs.* Genome Med, 2014. **6**(11): p. 90.
530 28. Wilgenbusch, J.C. and D. Swofford, *Inferring evolutionary trees with PAUP*.*
531 Curr Protoc Bioinformatics, 2003. **Chapter 6**: p. Unit 6 4.
532 29. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL) v4: recent updates*
533 *and new developments.* Nucleic Acids Res, 2019. **47**(W1): p. W256-W259.
534 30. Birkett, C.I., et al., *Real-time TaqMan PCR for rapid detection and typing of*
535 *genes encoding CTX-M extended-spectrum beta-lactamases.* J Med
536 Microbiol, 2007. **56**(Pt 1): p. 52-5.
537 31. Harris, P.N.A., et al., *Effect of Piperacillin-Tazobactam vs Meropenem on 30-*
538 *Day Mortality for Patients With E coli or Klebsiella pneumoniae*
539 *Bloodstream Infection and Ceftriaxone Resistance: A Randomized Clinical*
540 *Trial.* JAMA, 2018. **320**(10): p. 984-994.
541 32. Liakopoulos, A., D. Mevius, and D. Ceccarelli, *A Review of SHV Extended-*
542 *Spectrum beta-Lactamases: Neglected Yet Ubiquitous.* Front Microbiol,
543 2016. **7**: p. 1374.

544 33. Orencia, M.C., et al., *Predicting the emergence of antibiotic resistance by*
545 *directed evolution and structural analysis.* Nat Struct Biol, 2001. **8**(3): p.
546 238-42.
547 34. Jacoby, G.A., *AmpC beta-lactamases.* Clin Microbiol Rev, 2009. **22**(1): p.
548 161-82, Table of Contents.
549 35. Meini, S., et al., *AmpC beta-lactamase-producing Enterobacterales: what a*
550 *clinician should know.* Infection, 2019. **47**(3): p. 363-375.
551 36. Cheng, L., et al., *Piperacillin-Tazobactam versus Other Antibacterial Agents*
552 *for Treatment of Bloodstream Infections Due to AmpC beta-Lactamase-*
553 *Producing Enterobacteriaceae.* Antimicrob Agents Chemother, 2017.
554 **61**(6).
555 37. Grayson, M.L., et al., *Effects of the Australian National Hand Hygiene*
556 *Initiative after 8 years on infection control practices, health-care worker*
557 *education, and clinical outcomes: a longitudinal study.* Lancet Infect Dis,
558 2018. **18**(11): p. 1269-1277.
559 38. Giedraitiene, A., et al., *Prevalence of O25b-ST131 clone among Escherichia*
560 *coli strains producing CTX-M-15, CTX-M-14 and CTX-M-92 beta-lactamases.*
561 Infect Dis (Lond), 2017. **49**(2): p. 106-112.
562 39. Nicolas-Chanoine, M.H., X. Bertrand, and J.Y. Madec, *Escherichia coli*
563 *ST131, an intriguing clonal group.* Clin Microbiol Rev, 2014. **27**(3): p. 543-
564 74.
565 40. Hilty, M., et al., *Transmission dynamics of extended-spectrum beta-*
566 *lactamase-producing Enterobacteriaceae in the tertiary care hospital and*
567 *the household setting.* Clin Infect Dis, 2012. **55**(7): p. 967-75.
568 41. Tchesnokova, V., et al., *Pandemic fluoroquinolone resistant Escherichia coli*
569 *clone ST1193 emerged via simultaneous homologous recombinations in 11*
570 *gene loci.* Proc Natl Acad Sci U S A, 2019. **116**(29): p. 14740-14748.
571 42. David, S., et al., *Epidemic of carbapenem-resistant Klebsiella pneumoniae in*
572 *Europe is driven by nosocomial spread.* Nat Microbiol, 2019.
573 43. Lee, C.R., et al., *Global Dissemination of Carbapenemase-Producing*
574 *Klebsiella pneumoniae: Epidemiology, Genetic Context, Treatment Options,*
575 *and Detection Methods.* Front Microbiol, 2016. **7**: p. 895.
576

577

578

579 **Tables**

580

581 **Table 1:** Summary of antimicrobial resistance mechanisms among 288 clinical Enterobacteriaceae isolates retrieved from 258 patients admitted to a
582 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%)

583 [a]Antimicrobial susceptibility testing performed by automated method (VITEK 2®) and EUCAST breakpoints applied
584 [b]ESBL phenotypic testing performed by disc diffusion method
585 [c]DNA integrity was confirmed by 16S rDNA PCR [15], which forms part of the pentaplex assay. Three PCR negative *E. coli* strains encoded $bla_{\text{DHA-1}}$, one *E.*
586 *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
587 isolates had no β-lactamases identified despite whole-genome sequencing.